Thesis Ref. No. ___

ANTIGEN AND MOLECULAR DETECTION OF PESTE DES PETITS RUMINANTS VIRUS FROM DISEASE OUTBREAK CASES IN SHEEP AND GOATS IN ASOSSA ZONE, BENISHANGUL-GUMUZ REGION, ETHIOPIA

MSc Thesis

By

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MSc Program in Veterinary Microbiology

June, 2020
Bishoftu, Ethiopia

ANTIGEN AND MOLECULAR DETECTION OF PESTE DES PETITS RUMINANTS VIRUS FROM DISEASE OUTBREAK CASES IN SHEEP AND GOATS IN ASOSSA ZONE, BENISHANGUL-GUMUZ REGION, 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

By

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June, 2020

Bishoftu, Ethiopia

ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE
DEPARTMENT OF VETERINAY MICROBIOLOGY

As MVSc research advisor I hereby certify that I have read and evaluated this Thesis prepared under my guidance by **Tolessa Ebissa** entitled: **Antigen and Molecular Detection of Peste Des Petits Ruminants Virus from Disease Outbreak Cases in Sheep and Goats in Asossa Zone, Benishangul-Gumuz Region, Ethiopia**, I approved for submission to dissertation assessment committee.

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DEPARTMENT OF VETERINARY MICROBIOLOGY

As members of Examiners Board of the final MVSc open defense, we certify that we have read and evaluated this Thesis prepared by **Tolessa Ebissa** entitled: **Antigen and Molecular Detection of Peste Des Petits Ruminants Virus from Disease Outbreak Cases in Sheep and Goats in Asossa Zone, Benishangul-Gumuz Region, Ethiopia** and recommend that it is accepted as fulfilling the thesis requirement for the degree of Master of Veterinary Science in Veterinary Microbiology.

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DEDICATION

This thesis manuscript is dedicated to my Aunt who died during my MSc study program for unforgettable nursing and care she gave me with affection and love.

STATEMENT OF THE AUTHOR

First, I affirm that this thesis is my solely work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for Master's degree in Veterinary Microbiology at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers 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 award.

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Date of Submission: 8/6/2020

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ACKNOWLDEGMENTS

First of all, my heartfelt thank goes to Almighty God and his Mother for giving me health, strength, patience and protection.

I am very much grateful to my advisor Dr. Fufa Dawo for his encouragement, guidance, insight and constructive comments on this research work.

I extend my sincere appreciation to NAHDIC for their support and permission to attach me as a fellow student to their laboratory during my research work. I want to express my heartfelt thanks to Dr. Tesfaye Rufael, Dr. Getachew Abichu and Dr. Daniel Gizaw for their unforgettable hospitality and supports they gave me. It is a very good opportunity for me to forward my special thanks and respect to Mrs. Menbere, Mr. Abde Aliy, Mr. Damasa, Mrs. Tsion, Dr. Rediet, Dr. Dereje and Mr. Demeke for their unreserved help provided during the laboratory work. Especially I don't forget Mrs. Menbere, Mr. Abde and Mr. Damasa with their keen interest to share their rich and experienced technical know-how.

My gratitude also extends to Asossa regional animal health diagnostic laboratory staffs and lab technicians for their support during my field work especially Dr. Gebrehiwot, Mr. Yami and Mr. kebede for their unreserved help.

I sincerely thank Asossa and Bambasi district community in all locations of this study (herders and Professionals) for their warm welcome, homely accommodation and invaluable participation.

Last but not least, my family, my wife Temir Bushira and our beloved twin daughters Sifan and Sifenet Tolessa inspired all through my way with love, I thank you

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LIST OF ABBERVATIONS

AUIBAR	African Union Inter-African Bureau for Animal Resources
AZOA	Asossa Zone Office of Agriculture
CCPP	Contagious Caprine Pleuropneumonia
cDNA	complementary Deoxyribose Nucleic Acid
CDV	Canine Distemper Virus
CFSPH	Center for Food Safety and Public Health
CPE	Cytopatic Effect
CSA	Central Statistical Agency
DIVA	Differentiating Infected from Vaccinated Animals
DMEM	Delbuco's Minimum Essential Medium
DNA	Deoxyribose Nucleic Acid
ESCRT	Endosomal Sorting Complexes Required for Transport
FAO	Food and Agriculture Organization
FMD	Foot and Mouth Disease
GIS	Geographical Information System
IC-ELISA	Immune Capture Enzyme Linked Immunosorbent Assay
Kb	Kilo base
m.a.s.l	meter above sea level
MV	Measles Virus

LIST OF ABBRVATIONS (Continued)

NAHDIC	National Animal Health Diagnostic and Investigation Center
NMSA	National Metrological Service Agency
OD	Optical Density
OD_{NC}	Optical Density Negative Control
OD_{PC}	Optical Density Positive Control
OIE	Organization of International des Epizooties
PBS	Purified Phosphate Buffer Solution
PCR	Polymerases Chain Reaction
PPR	Pest des Petits Ruminants
PPRV	Pest des Petits Ruminants Virus
RNA	Ribose Nucleic Acid
rpm	revolution per minute
RPV	Rinderpest Virus
RT-PCR	Reverse Transcribing Polymerase Chain Reaction
s-ELISA	Sandwich Enzyme Linked Immunosorbent Assay
SLAM	Signaling Lymphocyte Activation Molecule
TAD	Trans-boundary Animal Diseases
TMB	Tetramethyl Benzidine
UAE	United Arab Emirates
UK	United Kingdom

LIST OF ABBRVATIONS (Continued)

USA	United State of America
USD	United State Dollar
VDS	Vero Dog SLAM cells
VNT	Virus Neutralization Test
VTM	Virus Transport Media

ANTIGEN AND MOLECULAR DETECTION OF PESTE DES PETITS RUMINANTS VIRUS FROM DISEASE OUTBREAK CASES IN SHEEP AND GOATS IN ASOSSA ZONE, BENISHANGUL-GUMUZ REGION, ETHIOPIA

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ABSTRACT

Peste des Petits Ruminants (PPR) disease is a severe, highly contagious and fatal viral disease of small ruminants causing a lot of production loss and mortality in Ethiopia. Limited serological and molecular reports indicated that the disease was highly prevalent in the study area and recently the data from the regional livestock agency indicated there was an improvement in vaccination coverage. Despite this, there was continuous occurrence of disease outbreak in the region. Thus, the aim of this study was to isolate and genetically identify recently circulating PPR virus (PPRV) by molecular tools from outbreak cases in small ruminants in the Asossa zone, Benishangul-gumuz regional state. Cross sectional study design were applied from November 2019 to April 2020 for investigation of the disease in outbreak areas. A total of 27 swab samples (22 nasal and 5 rectal swab) were purposively collected from clinically suspected animals and examined for the presence of PPRV by Immune capture Enzyme Linked Immunosorbent Assay (Ic ELISA) and a one-step Reverse Transcription Polymerase Chain Reaction (conventional and real time RT-PCR) assay. Of the clinical samples examined, 45.4% and 36.4% of the samples were positive for PPRV using Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Immune capture Enzyme Linked Immunosorbent Assay (Ic ELISA) respectively. Two out of twenty two PPRV

suspected sample was successfully isolated on Vero dog SLAM (VDS) cell line with the dog signaling lymphocyte activation molecule (SLAM) receptor expressed on the cell surface and confirmed with Ic ELISA and RT-PCR. As evidenced from clinical finding, virus isolation and molecular detection indicated PPRV was circulating in the area where all of the cases were associated with unvaccinated and newly introduced small ruminants from the neighboring region indicating the possibility of the virus spread to different districts in the region. Therefore, vaccination strategies and vaccine coverage should be improved and implemented especially in newly introduced sheep and goat. Further investigation should be done regarding the molecular epidemiology and genetic analysis of the virus circulating in the region.

Keywords: *Asossa Zone, Ethiopia, Goats and sheep, Ic ELISA, Molecular detection Outbreak Investigation and Peste des Petits Ruminants Virus*

1. INTRODUCTION

Goat and sheep breeds are numerous which are found in a variety of livestock production systems and adapt to different agro ecology in many parts of the world. Compared to large ruminant they provide vast product and service such as easily sold for cash, provide milk, meat, wool and their fast reproduction rate enables these animals essential for the herders or farmers where they are used as insurance against crop failure (Nottor, 2012; Abriham *et al*., 2018). In Ethiopia, there is about 30.70 million sheep and 30.20 million goats (CSA, 2017) which contribute to 25% of domestically consumed meat, 50% of domestic needs in wool, 40% of skin and 92% of the value of hide and skin exported to other countries. The annual production of sheep and goat meat is estimated as 56, 560 and 28, 650 tones, respectively (Waret-Szkuta *et al*., 2008).

Even though, small ruminants play an important role in food security and livelihood resilience in many parts of the world, there are several constraints reducing the productivity in this sector. Infectious disease is considered as a major restriction causing direct losses, such as death and decreased production, and indirect losses, such as export constraints. Peste des Petits ruminants disease (PPR) is among one of the most important diseases affecting small ruminant worldwide (Torsson *et al*., 2017; Hailegebreal, 2018). It is caused by *Small Ruminant Morbillivirus* or *Peste des Petits Ruminants Virus* which belongs to family *Paramyxoviridae,* subfamily *Orthoparamyxovirinae* and genus *Morbillivirus* (www.ictv.com, accessed on September 2019). In Ethiopia, it is among the most important disease of sheep and goats followed by *Contagious Caprine Pleuropneumonia* (CCPP) and Sheep and goat pox diseases that affect small ruminants entailing a huge economic loss and listed as trans-boundary disease of animals affecting the economy of the country (Befikadu and Endale, 2017).

The disease is an acute, highly contagious, and frequently fatal viral disease of sheep, goats and wild small ruminants. It is characterized by fever, mucopurulent ocular and

nasal discharges, necrotizing and erosive stomatitis, severe enteritis and pneumonia leading to death. PPR is a trans-boundary animal disease of significant economic importance, ranking among the top ten diseases affecting small ruminants (Gari *et al*., 2017). It is mainly transmitted through direct contact with secretions of infected animals causing high morbidity and mortality rate, reaching up to 100% and over 90% in naïve herds respectively. Mortality occurs between 5 and 10 days of onset of infection, with the few recovering animals developing strong lifelong immunity (Wang *et al*., 2013; Burns *et al*., 2019).

The disease is widely distributed across the sub-Saharan Africa, Middle East, Arabian Peninsula and the Indian subcontinent which causes serious economic losses and remains a major deterrent to a successful development of small ruminant production (El-Yuguda *et al*., 2009). In Ethiopia, the disease was suspected for first time in 1977 in goat herd in Afar region based on clinical evidence where the virus was later detected in 1994. The virus was subsequently identified and clustered in lineage III based on phylogenetic analysis of the virus (Roeder *et al*., 1994). Moreover, recently the emergence of lineage IV was reported by Muniraju *et al.* (2014) from male goats purchased from a market, though not substantiated at field level, has posed additional threat in the control of the disease and currently continuous occurrence of the diseases due to lineage IV in small ruminants in Ethiopia requiring further study in the molecular characterization of the spreading virus strains and phylogenetic analysis as indicated by Alemu *et al*., (2019) and Rume *et al*. (2019)

Previous report on sero-prevalence of the peste des petits ruminants disease showed that it was 75.7% in the Asossa zone as reported by Yalew *et al*. (2019) and more recently there is an improvement in the vaccine coverage to reduce the occurrence of the disease as information gathered from regional animal health agency. Despite this, the outbreak of the disease was still occurring during different seasons of the year in the region. Geographically, the region has shared border with north Sudan where there is market linkage between the areas; that may have possible contribution for the incursion of some trans-boundary animal diseases from neighboring country. Moreover, in this remote area

of the country so far no study well addressed on PPRV at molecular level; thus outbreak investigation and molecular detection of the virus has paramount importance. Therefore, the general objectives of this study was isolation and molecularly detect the virus causing of Pest des Petits Ruminants disease from outbreak cases occurring in different districts of Asossa zone.

Specific objectives

- To isolate *Peste des Petits Ruminants Virus* responsible for disease outbreak.
- To detect molecularly and identify the virus strain circulating in the study areas.

2. LITRETURE REVIEW

2.1. Historical Background

Peste des Petits Ruminants (PPR) disease was first reported by Gargadennec and Lalanne (1942) who investigated the syndrome in sheep (*Ovies aries)* and goat (*Capra hircus)* in Côte d'Ivoire, West Africa. Although *Rinderpest virus* can infect goats and sheep in Africa, most experimental infections are mild or sub-clinical and it was considered as severe forms of Rinderpest diseases in small ruminants but it was actually PPR. In the same way it was believed as Rindepest diseases when it was occur in goats in Sudan in East Africa but serological studies using specific antisera showed that the disease was actually PPR which represents the first isolate of the virus in East Africa (El Hag and Taylor, 1984; Saeed *et al*., 2009). Moreover, PPR was clinically suspected for the first time in Ethiopia in 1977 in a goat herd from Afar region, eastern part of the country (Pegram and Tereke, 1981) and later on confirmed in 1991 with cDNA probe in lymph nodes and spleen specimens collected from an outbreak in a holding near Addis Ababa (Roeder *et al*., 1994).

Peste des Petits Ruminants disease is emerging in new regions in the world causing significant animal and economic losses. Based on data available up until 2014, the virus is present in 65 countries with an additional 20 countries being classed as at risk (Jones *et al*., 2016; Burns *et al*., 2019). In recent years, PPRV has extended its boundaries southwards in Africa as far as southern Tanzania (2008), Zambia (2015) and the Democratic Republic of Congo and Angola (2012). PPR outbreak was also reported across North Africa including Tunisia (2006), Morocco (2008 and 2015) and Algeria (2011 and 2016). The first occurrence of PPR was reported in Georgia in February 2016 (Donduashvili *et al*., 2018). In East Asia, the virus spread to Tibet in 2007 and has recently been reported all over China (2013-2014) (Fakri *et al*., 2016).

However, following the global eradication of Rinderpest in 2011, the OIE and FAO have joined target PPR as the next animal disease to eradicate (Lancelot and De Almeida,

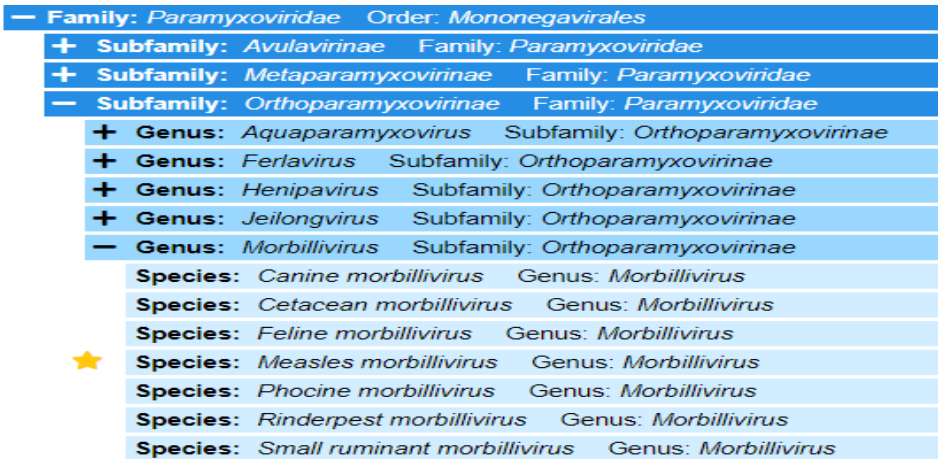
2016). A recent cost - benefit analysis study concluded that global eradication of PPR would see a return of $74 billion over 15 years (Jones *et al*., 2016). Over 90% of the world's small ruminant population is located in developing countries, providing nutrition, income from trade in animals and their products, improved economic stability and resilience for smallholder farmers. Eradication of PPR would likely provide considerable sustainability and welfare benefits to vulnerable communities across Asia, the Middle East and Africa (Burns *et al*., 2019).

2.2. Peste des Petits Ruminants disease

Peste des petits ruminants disease is a highly contagious infectious viral diseases that affect mainly small ruminants (sheep and goats) characterized clinically by fever, Muco-purulent ocular and nasal discharge, necrosis, pneumonia, ulceration of mucous membrane and inflammation of gastrointestinal tract that ultimately lead to severe diarrhea. It is one of strongest limiting factor in the development of the sheep and goat farming especially in developing countries where most of the people are rely on sheep and goat production (Abubakar *et al*., 2018).

2.3. Taxonomic classification of the virus

Small Ruminant Morbillivirus or *Peste des Petits Ruminants Virus* is belongs to family *Paramyxoviridae*, subfamily *Orthoparamyxovirinae* and genus *Morbillivirus* as described below in the figure 1.



The yellow star shows the type species or the most studied virus species of the family.

Figure 1: Taxonomic classification of PPRV

Source: (www.ictv.com, accessed on September 2019)

2.4. Etiology

Peste des Petits Ruminants disease is also known as goat plaque and the disease is caused by family of *Paramyxoviridae* of the genus *Morbillivirus* (www.ictv.com, accessed on September 2019). It is genetically closely related with *Rinderpest virus*, *Canine distemper virus* and *Human measles virus (*Özkul *et al.*, 2002*)*. There are four lineages of PPR viruses: lineage I-IV where lineage I and II viruses is found in West Africa, lineage III in East Africa, Arabian and Southern India, and lineage IV in the Middle East and Asia subcontinent but, recently linage IV which was distributed in Asian countries was detected in Africa (Muniraju *et al.*, 2014, Munir *et al.*, 2015). It is characterized by surviving at 60 ^{0}C for 1 hour, pH of 4 to10 and survives for long period of time with

chilled and frozen tissues but it is easily killed by alcohol, ether and detergents (Fentahun and Woldie, 2012; Chazya *et al*., 2015).

2.5. Genomic Organizations

The PPR virus has a non-segmented, single-stranded, negative-sense RNA genome which is 15,948 nucleotides in size (Adombi *et al*., 2016; Baron *et al*. 2016). The virus encode two non-structural proteins (C and V) and six structural proteins arranged in the order of nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin protein (H) and viral RNA-dependent polymerase (L) as described below in figure 2. The gene order is arranged in 3'-N-P(C/V)-M-F-H-L-5' and separated by inter genic region (Barrett *et al*., 2006; Mahapatra *et al*., 2006; Sen *et al*., 2010; Maganga *et al*., 2013; Baron *et al*. 2016).

It is a very polymorphic virus, although it is usually spherical which varies in size between 150 and 700 nm, with most particles measuring 500 nm (Diallo *et al*., 2019). The PPRV is composed of a helicoidal nucleocapsid surrounded by a lipoproteic envelope. Due to the presence of envelope, the virus is easily destroyed by lipid solvents and extreme environment since the virus is very delicate particularly outside of the host. The nucleocapsid is formed by a genome surrounded by three viral proteins, the most important of which is the nucleoprotein (N protein). The viral genome is simple, negative sense RNA strand virus that needs to be transcribed in to messenger RNA unless it cannot be translated directly into proteins. This stage is accomplished by an RNA-dependent polymerase complex, formed by two other nucleocapsid proteins: a polymerase-associated protein (P) (a phosphorylated protein) and a large polymerase protein (L) (Lefèvre and Diallo, 1990; Kumar *et al*., 2014).

H and F proteins enable the virus to become attached to the target cell and to release its nucleocapsid into the cytoplasm. The neutralizing antibodies produced by the infected host are directed against these proteins. Therefore, the genes of these two proteins might be of use in producing a PPR vaccine, with the help of genetic engineering. In addition to

the H and F proteins, there is a third viral envelope protein which coats its inner surface: the membrane protein (M). This provides the link between both external glycoproteins and the nucleocapsid and plays an important part in virus formation. N, P, L, H, F and M proteins are structural proteins because, together with the genome and the envelope, they constitute the viral particle. There is a seventh viral protein (C protein) which differs from the others in being non-structural; it is found only in cells infected by the virus. Its exact function is not yet known (Lefèvre and Diallo, 1990).

Based on the molecular characterization mainly on N and F genes, strains of PPRV can be grouped into four lineages, which are genetically distinct from each other but serologically monotypic (Boussini *et al*., 2016; Liu *et al.,* 2017). These includes Lineage I and II which were isolated from west Africa countries, lineage III were isolated from east Africa and lineage IV were isolated from middle east and Asian countries (Anees *et al*., 2013; Niyokwishimira *et al*., 2019). It is still unclear whether differences between lineages are merely reflecting geographical speciation or if they are also correlated to pathogenicity variability between isolates (De Nardi *et al*., 2012).

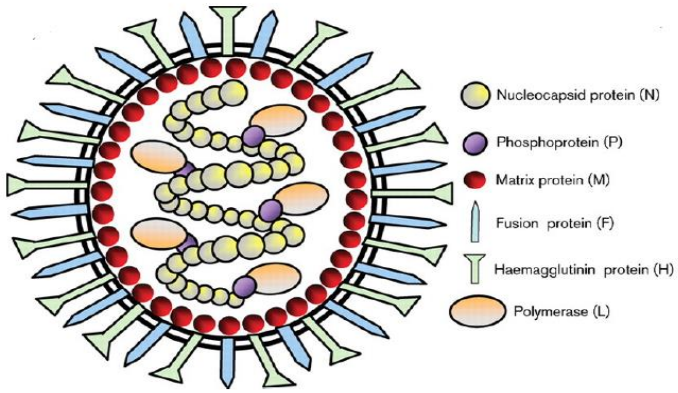


Figure 2: A schematic diagram of *Morbilivirus* virion structure
Source: Banyard *et al*. (2010)

2.6. Viral replication

2.6.1. Attachment and Entry

The replication cycle for different *Paramyxovirus* is similar which start with the attachment of the virus on the cell surface and membrane fusion to release a genome into the cell cytoplasm that plays an important role in pathogenesis and susceptibility of host (Kumar *et al*., 2014). The H protein is responsible for the attachment of the virus to the cell surface through recognition and binding to host cell receptor molecules such as salicylic acid, immune cell marker signaling lymphocyte activation molecule (SLAM)/CD 150 and epithelial cell receptor Nectin-4 (Baron *et al*., 2016; Yang *et al*., 2018; Qi *et al*., 2019).

SLAM is exclusively expressed on immune cells (lymphocyte, macrophages and dendritic cell surface), while Nectin-4 is the epithelial cell receptor, but none of the receptors are expressed on other than their specific cells. Existence of these two different receptors showed that tissue preference of the virus for lymphoid cells and epithelial cells which render the virus both lymphotropic and epitheliotropic affinity. The first receptor, SLAM, is certainly the most important, when the virus infects the host through the respiratory tract where it is taken by macrophages and dendritic cells and transported to local lymph nodes for multiplication (Birch *et al*., 2013; Kumar *et al*., 2014; Mantip *et al*., 2019). The second cellular receptor, Nectin-4, plays an important role for dissemination of *Morbillivirus* throughout the body by facilitating amplification and subsequent release of the virus via different secretions (exit receptor). Attachment of the H protein to receptors activates the fusion activity of the F protein, enabling a fusion of the viral envelope with the host cell membrane and release of the viral genetic material into the cell cytoplasm (Parida *et al*., 2015).

2.6.2. Transcription and Replication

Replication of *Morbillivirus* is solely takes place in cytoplasm of the host cells. In infected cells a viral genome encapsidated by N protein to form ribonucleoprotein that containing P and L proteins to make up minimum replicative units and protect the virus from host cell RNAse. When the virus is released in to cytoplasm of host cells it is acted upon by viral polymerase complex which binds to the genome promoter and start transcribing short leader RNAs. The polymerase works its way across the genome transcribing each gene in turn falling off at each inter genic region. The dissociation of the polymerase at each inter genic region across the genome leads to the build-up of a transcriptional gradient as the polymerase can only commence transcription at the genome promoter. The mRNAs produced are 5' methylated and 3' poly-adenylated by the viral polymerase and are translated by host cell machinery. At a certain time point post-infection, the polymerase complex switches its action from the production of mRNA to the production of a full length positive sense RNA. This switch is thought to be linked to the accumulation of viral proteins within the host cell. Following the production of a full length antigenome (+ve) RNA, the polymerase now binds to the antigenome RNA at the antigenome promoter (30) and generates nascent full length negative sense genomes. The synthesis of viral components within the cell eventually leads to viral egress from the host cell (Parida *et al*., 2015).

2.6.3. Virus Assembly and Release

The process of assembly and release of the morbillivirus is similar to other enveloped viruses which involves the formation of viral particle when all structural components of the virus are made. It includes viral glycoproteins and viral RNPs which are assembled at the selected sites on the membrane where the virions can be bud and pinch off to achieve the particle release that allow the transmission of infections to new cells and hosts. The viral M protein plays crucial role in the assembly and release by serving as an adapter to link together structural components of the virions (RNP core and viral glycoproteins) and cellular membranes. In addition to the M proteins, H/HN, F and C facilitate in the

assembly and budding process of the virus. Moreover, Enveloped viruses may not encode all of the machinery required for efficient budding; rather it is also supported by several host factors in release of the virions such as endosomal sorting complexes required for transport (ESCRT) and ubiquitin. *Paramyxovirus* RNPs interact with the M protein under the plasma membrane and buds via the ESCRT complex (Kumar *et al*., 2014).

2.7. Physiochemical properties of the virus

The molecular weight of the genome is $5.8x10^6$ while the diameter of the virion measures about 150-300 nm. The virion is very sensitive to heat, lipid solvents, non-ionic detergents, formaldehyde and oxidizing agents. The virus is usually destroyed when exposed to temperature of 50 ^{0}C for 60 minutes or 37 ^{0}C for 2 hours. However, it survives for long periods in chilled and frozen tissues (Organization of International des Epizooties, 2013; Dejene, 2016). The PPR virus is also sensitive to low pH, being destroyed after death of the animal by the low pH which accompanies rigor mortis particularly it loses its activity at pH below 4 or above 11 however, the virus is stable at pH of between 5.8 and 9.5 (Organization of International des Epizooties, 2013).

2.8. Immunosuppression

Peste des Petits Ruminants virus is highly lymphotropic that cause infection of lymphoid tissues that result in a profound immunosuppression by inducing leucopoenia and reduced antibody responses (Adombi *et al*., 2011; Pope *et al*., 2013; Latif *et al*., 2018). The virus can cause immunosuppression both in vaccinated and infected animals where the most virulent strain of the virus cause marked immunosuppression, whereas vaccination only induces a transient leucopoenia with no significant effects on the immune response (Rajak *et al*., 2005). Leucopoenia is observed generally from the fourth day post-infection and may revert depending on progression of disease (Baron *et al*., 2014; Herbert *et al*., 2014). In addition, some literature show that CD4+ cells were found to decrease in number from day four of post-infection where the proportion of CD8+ cells

were remain unchanged initially but, it increased by the seventh days of post-infection (Rojas *et al*., 2014; Rojas *et al*., 2015).

2.9. Epidemiology

2.9.1. Geographic distribution

Peste des Petits Ruminants disease is identified for the first time in the early 1940s in Cote d'Ivoire which has steadily expanded its geographical distribution beyond its original endemic region of West Africa. In deed there is dramatic and significant expansion of the disease over the past 15 years covering large parts of Africa, central Asia, south Asia and East Asia where it is recently endemic for the diseases as seen in figure 3. Currently the diseases is covering around 70 countries of the world and suspected as another 50 countries are at high risk of getting the diseases. Out of these infected countries, more than 60% are in Africa (including North Africa) whereas the remaining is in Asia (South East Asia, China, South Asia and Central Asia/ West Eurasia including Turkey) and the Middle East. However, 48 countries were recognized as PPR free by the OIE where these countries are historically free areas are mostly found in the Americas and Europe (OIE and FAO, 2015).

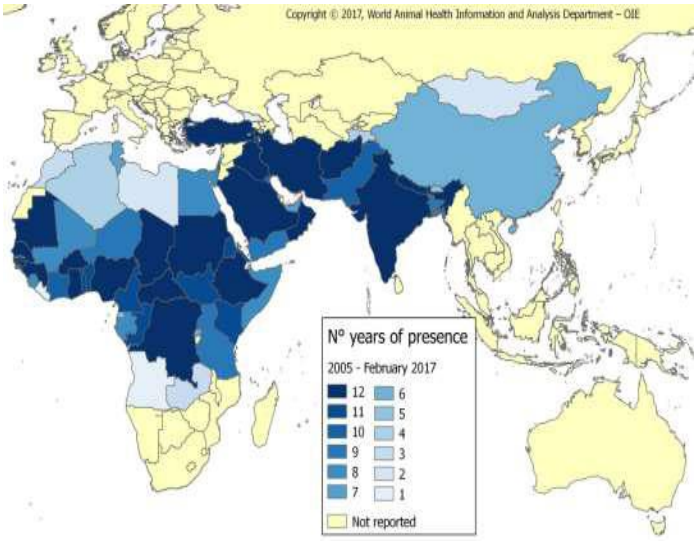


Figure 3: Map of Global incidence of PPR from 2005–2017.

Source: Bakkouri (2017)

2.9.2. Distribution of PPRV according to molecular diversity

The current molecular characterization or phylogenetic analysis based on partial sequencing of the F and N gene classified the virus into four genetically distinct lineages (Forsyth and Barrett, 1995; Couacy-Hymann *et al*., 2002). The lineages are generally correlated with geographic distribution of the virus. PPR viruses belonging to lineages I and II have been isolated exclusively from west and central African countries. Lineage III has been isolated from eastern Africa and Arabian Peninsula, lineage IV has been isolated in Asia, Middle East and more recently in northern Africa as shown below in figure 4 (Munir *et al*., 2012a; Shahriari *et al*., 2019). Nowadays, in a large zone of Africa, lineage IV is slowly replacing PPRV lineage III. The constant increase of the disease with the lineage IV incidence in north and east Africa and Asia indicate the virulence of the virus. Moreover, the presence of the two African lineages in Asia beside a distinct Asian lineage indicates trade as a factor for the disease spreading (Kwiatek *et al*., 2011; Libeau *et al*., 2014; Muniraju *et al*., 2014).

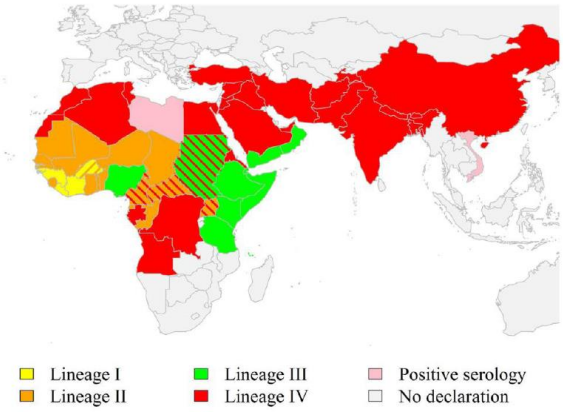


Figure 4: Map showing the molecular distribution of the four PPR virus (PPRV) lineages. Source: Albina *et al*. (2013).

2.9.3. Host species affected

The diseases affect small domestic ruminant, wild ruminants and camel where sheep and goats are clinically highly affected however, goats are considered more susceptible than sheep (Njue *et al*., 2018). Wild ruminants such as antelope, buffalo, hippotraginae, tragelaphinae, nigale, laristan sheep, dorcas gazelles, Nubian ibex and gemsbok are susceptible to PPR and potential source of infection for domestic animals (Chauhan *et al*., 2009; Kinne *et al*., 2010; Lembo *et al*., 2013; Munir, 2013a). The virus seroprevalence is also has been reported in cattle, buffalo and pigs but there was no observable clinical signs have been seen in these animals except in camels where these species are considered as dead end reservoirs of the diseases (Abubakar *et al*., 2015a; Kardjadj and Luka, 2016).

Clinical signs and death have been reported in experimentally infected calves and the virus has been isolated from domestic buffalo in India (Zakian *et al*., 2016). There are many reports that indicate that camel is infected with PPRV where the isolates from camels in epizootics outbreak in Ethiopia indicates as the diseases occur in these animals (Megersa *et al*., 2012). Further the virus is isolated and clinical case were reported from camel in Sudan by Khalafalla *et al*. (2010) and Kwiatek *et al*. (2011) and iran by Zakian *et al*. (2016). The virus was also isolated from wild goat and Bharals from Iran. Dorcas gazelle, Thomson's gazelle, Gemsbok and Ibex are also susceptible species (Kumar *et al*., 2017). Moreover, the virus was isolated from Sindh ibex in Pakistan by PCR and immune capture ELISA (IC ELISA) (Abubakar *et al*., 2011).

2.9.4. The role of other species in PPRV epidemiology

There are numerous reports of PPRV infection in species other than domestic sheep and goat such as wild sheep and goat, buffalo, gazelle, wildebeest, cattle, camel (Mahapatra *et al*., 2015; Woma *et al*., 2015; Abubakar *et al*., 2017) and recently even dogs (Ratta *et al*., 2016). This is important from transmission perspective of the diseases because of continuous existence of transhumance and pastoralism among sheep and goat herders.

However, few of the report discriminate species that can be infected sub-clinically, seroconvert but do not shed the virus, species that develop diseases and shed infectious virus and species in which the infection is clinically in apparent but the animal remain infectious and shed the virus. It is important that this observation has to be extended robustly to correlate the pathogenesis, antibody response and virus excretion. However it is difficult to determine the level of the virus excreted which has challenge in addressing the associated risk factors of PPR control and eradication campaigns (Baron *et al*., 2017).

The presence of the disease in wildlife is another area where additional research is needed. The disease was diagnosed in several wildlife populations such as Argali (Ovis ammon) in China; Afghan Markhor goat (Capra falconeri) in UAE; Arabian gazelles (Gazella gazelle) and Arabian mountain gazelles (Gazella gazella cora) in UAE; Bharals (Pseudois nayaur) in China; Barbary sheep (Ammotragus lervia) and Bushbucks (Tragelaphus scriptus) in UAE; Capra ibex (Capra ibex sibirica) in China; Dorcas gazelles (Gazella dorcas) in Saudi Arabia and Sudan; Goitered gazelle (Gazella subgutturosa) in China, Tanzania, Turkey and Sudan; Ibex (Capra ibex) in China; Impala (Aepyceros melampus) in Tanzania and UAE; Nubian ibex (Capra nubiana) in UAE; Wildebeest (Connochaetes gnou) in Tanzania, White-tailed deer (Docoileus virginianus) in USA; Wild goat (Capra aegagrus) in Iraq and Iran; Wild sheep (Ovis orientalis) in Iran and Lion (Panthera leo persica) in India. The presence of this virus in wide host range indicates that it has significant effect on the eradication program of the diseases (Dou *et al*., 2020).

2.9.5. Mortality and morbidity

The diseases causes high morbidity and mortality but sheep suffer less clinical diseases when compared with goats which is assumed to be because of sheep has some innate resistance to the diseases (Abubakar and Irfan, 2014). The morbidity and mortality rates of PPR can be up to 100% in severe outbreaks but in milder outbreaks, mortality rate may be reduced to 50% while morbidity rate still remains high in both cases (Organization of International des Epizooties, 2012). Mortality rate is high in the susceptible young

animals (4-8 months), animal with poor nutritional status, Stress and concurrent parasitic and bacterial infections also enhance the severity of the disease (Jilo, 2016). Super infections with other bacterial, viral or parasitic agents may increase mortality up to 100% (Mokhtari *et al*., 2017).

2.9.6. Transmission

The disease is mainly transmitted through respiratory route by aerosols and contact between infected and health animals. The infected animals are important source of transmission during incubation periods, subclinical cases or before the onset clinical signs for animals such as sheep, goat, camel, cattle and pigs. Animals affected by PPR shed the virus in exhaled air, in secretions and excretions from natural orifices approximately 10 days after the onset of fever (Shichibi *et al*., 2017). Sheep and goats are the primary hosts for PPRV with a few reports of disease outbreaks in camels. Cattle, buffalo and pigs develop a subclinical infection with PPRV, but they are not capable of excreting virus thus contributing to the epidemiology of the disease (Parida *et al*., 2016).

2.10. Clinical signs

Peste des Petits Ruminants virus has a tropism for epithelial and lymphoid cells. The incubation period is typically 4–6 days, but may range between 3 and 10 days. The clinical disease is acute in nature characterized by having fever (up to 41 ^{0}C) that can last for 3–5 days; pneumonia, coughing, plural rales, abdominal breathing, depression, anorexia, develop a dry muzzle with erosive to necrotic lesions, serous oculonasal to mucopurulent discharges that can persist for around 14 days and death. Moreover, it can also affect gastrointestinal tract causing Watery blood-stained diarrhea in the later stage of the diseases. (Organization of International des Epizooties, 2008, 2012; Albina *et al*., 2013; Balamurugan *et al*., 2014).

2.11. Pathogenesis

Pest des peptits ruminants virus is lymphotropic and epitheliotropic which induce sever damage in the organ rich in lymphoid and epithelial tissues (Parida *et al*., 2019). The natural route of infection of these viruses is through respiratory system. Following infection by nasal route the virus is taken up by the immune cells of the respiratory mucosa, alveolar macrophages and dendritic cells that migrate to the T-cell rich areas of lymphoid organs including tonsil for replication before entering to general circulation. Then the virus can spread to epithelial cells and digestive tract sites where predominant site of viral replication can occur along with the lymphoid tissues. Infection of those cells resulted in the development of necrotic lesions in lymphoid and epithelial cells. This destruction of lymphoid tissue causes lymphopenia and profound immune suppressive effects on the host thus, increase the susceptibility of the host to other opportunistic microorganisms and mortality (Diallo *et al*., 2019).

2.12. Diagnosis

2.12.1. Postmortem

The most common gross lesions observed during postmortem examination were enlarged and edematous lungs with observable rib imprints on the pleural surface, erosive and ulcerative stomatitis with multiple 1 to 2 mm erosive lesions and ulcers in buccal cavity majorly affecting tongue, soft and hard palates. Pathological changes were also observed in small intestine that varied from distended intestinal loops with watery contents and hyperemic mucosa containing blood tinged intestinal contents and presence of enlarged and edematous mesenteric lymph nodes as reported by Abedalkhader *et al*. (2020) Moreover, the most severe lesions are seen in the large intestine, with congestion and "zebra stripes" of congestion on the mucosal folds posterior parts of colon (Khaliq *et al*., 2020). Congestion and enlargement of spleen and lymph node were occurs with erosive lesions in vulva and mucous membranes of the host (Kozat and Sepehrizadeh, 2017).

2.12.2. Histopathological Findings

Histopathologically, necrotic stomatitis, necrotic tonsillitis, fibrinohemorrhagic enteritis, proliferative interstitial pneumonia and suppurative broncho-interstitial pneumonia can occur. The presence of multi-nucleated giant cells or syncytia, infiltration of the alveoli by mononuclear cells and Lesions in the large intestines revealed proliferation of goblet cells, congestion of blood vessels, edema and infiltration of lamina propria by inflammatory cells (Chauhan *et al*., 2009; Rahman *et al*., 2011; Kihu *et al*., 2014). In addition, non-purulent enteritis with congestion and infiltration of large mononuclear cells in the lamina propria and loss of lining epithelium were seen in the intestine. Loss of surface epithelium and congestion of rumen, Sloughing of surface epithelium and infiltration of mononuclear cells in the trachea, inflammatory exudates containing large amounts of fibrin and mononuclear cells accumulated over a considerable area of the parenchyma, the alveoli and interstitium of lung and depletion of lymphocytes and infiltration of macrophages in lymph nodes are some of the pathological changes due to the diseases (Rahman *et al*., 2011).

2.12.3. Pen-side test

A rapid immunochromatographic strip test has been developed and shows extremely high sensitivity and specificity to diagnose the diseases. This test can confirm or disprove the presence of virus in a location in real time, thus enabling effective surveillance to underpin targeted vaccination (Mariner *et al*., 2016). These tests are based on so-called lateral flow technology. Conjunctival, nasal or oral swabs are taken from suspect animals, the swabs are rinsed with buffer and this buffer is applied to one end of a chromatographic strip. The sample mixes with colored beads coated with a specific MAb that recognizes PPRV antigen. Buffer flow moves the beads along the chromatographic strip. If the sample contains PPRV antigen captured by the swab, this binds to the beads, and the antigen and beads complex is then captured by a line of anti-PPRV MAb part-way along the strip, making a colored line to indicate a positive result. In the absence of PPRV antigen, no beads are bound by the test line. The tests take 20 minutes and require

no additional equipment. Both tests have been validated against PPRV isolates from all four lineages, and show sensitivity similar to IC-ELISA, and 100% specificity in laboratory testing (Organization of International des Epizooties, 2019).

2.12.4. Virus isolation

Among all the methods, isolation of the virus remains the ‘‘gold standard’’ for diagnosis of PPR. It can be isolated and grown in vitro in primary bovine and sheep cells as well as established cell lines such as Vero (African green monkey kidney) cells and Marmoset B-lymphoblastoid-B95a cells (Muthuchelvan *et al*., 2015). Virus isolation cannot always be done as routine diagnostic assays because they are time-consuming and cumbersome and require cell culture facilities, and they are not as sensitive as RT-PCR (Luka *et al*., 2011) The virus manifests with specific cytopathic effect (CPE) after 3–5 days of infection, which include initial rounding of the infected cells in grape-bunch-like clusters, followed by vacuolation, granulation of the cell cytoplasm, fusion of the monolayer cells and formation of syncytia, which are characteristics of PPRV (Elsheikh *et al*., 2019).

2.12.5. Virus neutralization test (VNT)

Virus Neutralization Test (VNT) was performed for confirmation and differentiation of RP and PPR viruses, either in tubes or in micro-titer plates (micro-VNT) for the detection of PPRV antibodies in serum samples. Differential neutralization test is one of the important means to distinguish RP and PPR viruses and a micro-VNT has been employed to measure the infectivity titer of RPV and PPRV in calf kidney, sheep kidney and Vero cells. Although PPRV clinically and antigenically related to RPV, PPRV is distinguishable serologically not only from RPV but also from MV and CDV. Parallel titration of unknown sera for different antibody responses against PPR and RP viruses has been shown to detect specific etiology in earlier days (Balamurugan *et al*., 2014).

2.12.6. Molecular detection techniques

RT-PCRs have been reported for detection and differential diagnosis of RP and PPR viruses in clinical specimens. The PCR techniques have been developed targeting F gene (Forsyth and Barrett, 1995), N gene (Couacy-Hymann *et al*., 2002; George *et al*., 2006), M gene (Balamurugan *et al*., 2006, George *et al*., 2006) and H gene (Balamurugan *et al*., 2010, 2014) and used for specific detection of PPRV from clinical samples. A PCR for PPRV/RPV using PPR virus-specific external protein F gene primer (F1 and F2) (Forsyth and Barrett, 1995) has gained great importance for differential diagnosis and for epidemiological studies. However, this assay may not always be suitable for diagnosis of every virus strain, variant or isolate, as changes at the 3' end of the primer binding sites, as a result of variation between strains in the immunogenic protein coding region, may yield a false-negative result. A two-step RT-PCR has been shown to useful for the rapid detection of virus specific RNA in the samples submitted for laboratory diagnosis (Couacy-Hymann *et al*., 2002, George *et al*., 2006). Further, PCR strategies targeting M and N gene have been developed for detection and differentiation of PPRV in sheep and goats. In a similar direction, a RT-PCR assay based on the M and N gene has been developed (Balamurugan *et al*., 2006; George *et al*., 2006), which was employed successfully for the direct detection of PPRV in the clinical samples.

The one-step multiplex RT-PCR based on N and M genes for use in differential diagnosis of PPR from RP in a single reaction, which has the potential to replace the existing F gene based PCR for diagnosis of PPR was developed which enhance the existing diagnostic technique (Balamurugan *et al*., 2006). A highly sensitive N-gene based RT-PCR-ELISA for the detection and differentiation of PPRV has also been developed using DIG labelled RT-PCR product (Saravanan *et al*., 2004). As an improvement over conventional PCR approaches, recently the real-time RT-PCR techniques targeting either N or M gene using TaqMan hydrolysis probe, SYBR Green with melting curve analysis have been in use for rapid, highly sensitive and specific detection and quantitation of PPRV. Earlier, N gene based one step TaqMan real-time RT-PCR has been developed for

detection of PPRV nucleic acid in the clinical samples with high sensitivity (Bao *et al*., 2008)

2.13. Treatment, control and prevention

Supportive treatment of sick animals with broad spectrum antibiotics to prevent secondary bacterial complications is needed. In early stages of the diseases sick animals should be kept isolated and given hyper-immune serum with extensive fluid therapy to cope up the dehydration due diarrhea. Lesions around nostrils, mouth and eyes should be cleaned with provision of proper nursing and caring (Abubakar and Irfan, 2014).

In non-endemic areas import control, movement restriction and ring vaccination of high risk population is important and the diseases can be eradicated with the combination of quarantines, euthanasia of infected and exposed animals and cleaning and disinfection of contaminated premises (Banyard *et al*., 2010; Kamel and El-Sayed, 2019). Inactivation of the virus in the environment has also important role since the virus cannot remain viable outside of the host for more than four days and burning or burying of the infected carcass is also helpful in eradication of the diseases. Further care should be taken to prevent the spread of the virus to the potentially susceptible wild animal populations such as deer, gazelles, wild sheep and goats (Center for Food Security and Public Health, 2015).

In endemic the diseases is mostly controlled by vaccination which provide the animal long term immunity and animals recovered from the diseases can develop good immunity that can persist for at least four years. To help protect susceptible wildlife and captive wild animals in endemic regions, they should not be allowed to have contact with sheep and goats. It might also be possible to vaccinate exotic species, but there is currently little information on the safety and efficacy of sheep/goat PPR vaccines in other animals. One outbreak among Sindh ibex at a wild animal park seemed to be controlled by vaccinating domesticated small ruminants and disinfecting common water sources in nearby villages (Center for Food Security and Public Health, 2015).

2.14. Economic Impact of PPR Disease

The impact of PPR disease on small ruminant productivity includes mortality, loss of milk, meat, fibers and hides, weight loss, impaired growth, and abortion. As PPR is an epidemic disease, its impacts are clustered at the household and community levels. When it occurs, PPR results in shortages of milk and meat to feed the family, as well as lack of animals for sale to provide cash to purchase cereals and other foodstuffs, basic household necessities, as well as essential health and educational needs. Fewer animals are available for social purposes such as loans and gifts, marriages, and funerals, and thus important social events may be postponed for months or years. Livelihood impacts that affect the well-being of communities are hard to quantify, thus they are among the most important cited by communities (Mariner *et al*., 2016; Kozat and Sepehrizadeh, 2017).

PPR is present in around 70 countries in Africa, the Middle East and Asia, threatening more than 1.7 billion of the total global population of 2.1 billion sheep and goats, as well as the livelihoods, food security and nutrition of more than 330 million people in these regions mainly poor farming communities that rely solely on small ruminant production for their survival. Another 50 countries are at risk of incursions of the disease from neighboring areas, threatening an additional 167 million sheep and goats. PPR not only affects the families who raise sheep and goats, but also the well-defined and complex value chains that these production systems supply. In the worst case scenario, PPR outbreaks can decimate over 90 percent of healthy flocks of sheep and goats. In endemic areas, the disease is insidious, affecting the development of young animals and the ability of adults to fight bacterial diseases, limiting the development of healthy and thriving herds and flocks of goats and sheep. Economic losses caused by PPR strike at the heart of vulnerable livelihoods as well as national and regional livestock production. Countries have experienced yearly losses ranging from tens to hundreds of millions of dollars (Organization of International des Epizooties and Food and Agriculture Organization, 2015).

PPR is currently considered as one of the main trans-boundary animal diseases (TAD) that hampers livestock production in many developing countries particularly in West and East Africa and South Asia, notifiable to the Office of International Epizootics. In all, 62.5% of the global domestic small ruminant population is at risk of PPR. The diseases cause estimated loss of USD 1.45 to 2.1 billion every year (Folitse *et al*., 2016).

In Ethiopia the economic importance of PPR is primarily due to its highly contagious nature, with a case fatality rate as high as 100% in sever outbreaks. In milder outbreaks, morbidity is still high but the mortality rate may be closer to 50%. This is of particular concern for the economics of small rural farms, where sheep and goats are reared as the sole source of income. Moreover, PPR is most prevalent in countries like Ethiopia where the peoples are relay heavily on subsistence farming of small ruminants for trade and food supply (Munir, 2013b; Befikadu and Endale, 2017). In Ethiopia, FAO estimated that losses associated with PPR reached an average of USD 375 per flock per year, for an average flock size of 143 small ruminants (an average loss of more than USD 2 per animal) (African Union Inter-African Bureau for Animal Resources, 2015).

2.15. Status of Peste des Petits Ruminants diseases in Ethiopia

In Ethiopia, Clinical PPR was suspected in 1977 in afar region, east of the country. Clinical and serological evidence of its presence confirmed in 1991 in Addis Ababa. Gelagay has reported that 14.6% of sheep sampled along 4 roads from Debre Berhan to Addis Ababa were seropositive for PPR. Waret-Szkuta *et al*. (2008) has also reported an overall sero-prevalence of 15.3% in Afar, 4.6% in Amhara, 8.0% in Benishangul Gumuz, 1.7% in Oromia, 1.8% in SNNPR and 21.3% in Somalia regions of Ethiopia. Most recently, PPR prevalence of 47.5% and 48.43% has been reported from different parts of Tigray and Oromia regions of Ethiopia (Waret-Szkuta *et al*., 2008).

Based on three studies conducted in Afar and Gambella regions at different times, the prevalence of PPR appears to be increasing in both sheep and goats (Abraham *et al*., 2005; Waret-Szkuta *et al*., 2008; Megersa *et al*., 2011). The prevalence of PPR was

15.3% in 1999 (Waret-Szkuta *et al*., 2008), 16% in 2001 (Abraham *et al*., 2005), and 38.3% in 2010 in Afar (Megersa *et al*., 2011). Similarly, it was 23% in sheep and 22% in goats in 2001 (Abraham *et al*., 2005), and 31.0% in sheep and 26.3% in goats in 2010 in Gambella (Megersa *et al*., 2011). More recently, report on sero-prevalence of the peste des petits ruminants disease showed that it was 75.7% in the Asossa zone (Yalew *et al*., 2019) and also an overall seroprevalence record of 30.9% from sheep and goat in pastoral and agro-pastoral area of afar and Gambella region of Ethiopia has been reported (Hailegebreal, 2018).

Data on molecular study showed that peste des petitis ruminants virus was detected in 1991 and subsequently the isolate was genetically determined as lineage III in 1994 (Roeder *et al*., 1994). Thereafter PPR disease outbreak was continued to occur in the country with the emergency of new lineage IV in 2010 as reported by (Muniraju *et al*., 2014). More recently, molecular epidemiology of PPRV show that lineage IV was continuously reported circulating in different parts of Ethiopia causing a lot of outbreaks, production losses and mortality (Alemu *et al*., 2019; Rume *et al*., 2019).

2.16. Opportunities Presented for Eradication of PPR

The epidemiology and the biological nature of PPRV is very close to the *Rinderpest virus* this enable to control and eradicate the diseases in the same ways as that of *Rinderpest virus* diseases. There are several aspects that favors the eradications of the diseases such as presence of only one serotype which is believed to provide cross protection within strains of different lineages, absence of carrier state that act as possibly source of infection, development of life long immunity after vaccination, the dependency of the virus on close contact between animals for effective transmission, the inability of the virus to survive for long period of time outside of the host and presence of appropriate diagnostic tools are the most important indicators that possibly enable for effective control and eradication of the diseases (Kumar *et al*., 2013; Munir *et al*., 2013c; Njeumi *et al*., 2020).

2.17. Gaps and research needs

Peste des petits ruminants diseases is challenged with many constraints that need improvement through research of which DIVA vaccine (with associated laboratory tests to discriminate vaccination from infection) would be useful tools in improving the efficiency of control measures. In particular, a DIVA vaccine would facilitate surveillance for actual disease during an ongoing vaccination campaign (Chauhan *et al*., 2014; Getachew *et al*., 2018). Furthermore, development and technology transfer of an efficacious thermo-stable vaccine against PPRV remains an area for further research (Food and Agriculture Organization, 2013; Banyard and Parida, 2016).

Epidemiological research which will include antibody/virus dynamics in populations, estimation of the basic reproductive rate and virus virulence determinants are also needed. A major outstanding requirement is to improve our epidemiological knowledge of the distribution of PPR and particularly to improve our knowledge of the role played by wildlife in the transmission and potential maintenance of PPRV in the environment in order to be able to initiate successful control strategies (Fine *et al*., 2020).

Though a good PPR vaccine is available, the definition and implementation of a relevant vaccination strategy and vaccination monitoring might be tricky. Among others, the questions of the quick turnover in sheep and goat populations, accessibility of these populations for vaccination and monitoring, intensity of small ruminant trade and transhumances, have to be considered (Yirga *et al*., 2020). In addition, spatial and temporal heterogeneities in sheep and goat population density and dynamics, as well as differences in breed receptivity and sensitivity to PPR virus, make it difficult to define a priori the most efficient vaccination strategy (Albina *et al.*, 2013; Padhi and Ma, 2014).

2.18. Challenges in eradication of the disease

The control and eradication of PPR disease can be challenged by different factors that may possibly hinder the success of the eradication strategies. These includes high population and high turnover rates of small ruminants (Yirga *et al*., 2020); low vaccine coverage to achieve sufficient flock immunity levels to interrupt virus transmission (Diallo, 2006); perceptions of low individual economic value of goats/sheep relative to the large ruminants masking the high social value of small ruminants; highly scattered geographical distribution of small ruminants that could complicate the vaccine delivery process; high mobility of small ruminants compared to cattle within a country and between countries; the expected higher cost of PPR eradication compared to that of Rinderpest; high burden of small ruminant diseases, lack of effective animal identification and traceability systems and weak veterinary services in many countries (African Union InterAfrican Bureau for Animal Resources, 2015; Fournié *et al*., 2018; Nkamwesiga *et al*., 2019).

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted in Asossa and Bambasi district of Benishangul-gumuz regional state, western Ethiopia. Asossa district is located at 668Km North West of Addis Ababa which covers an area of 2317 km having altitudinal range of 580-1544 meter above sea level. It is located at 10^0 04' north latitude and 34^0 31' 59" east longitudes (Figure 5). The average annual rainfall is 900-1200 mm with unimodal type of rainfall that occurs between April and October and average temperature ranges from 19 ^{0}C to 34 ^{0}C (National Meteorological Services Agency, 2015). The area is characterized by mixed type of farming system having 31,632 cattle, 29,631 goats, 19,922 sheep, 6,877 equines (donkey and mules) and 91,460 poultry of the livestock population (Asossa Zone Office of Agriculture, 2018).

Bambasi district is located 9^0 45' N and 34^0 45' east. The altitude of the area ranges from 1100-1450 m.a.s.l. bordering Dabus river system to east direction. Topography of the area is marked by hill, sleep slopes and flat surface of the land. The district has a sub-humid climate with moderate hot temperature with less variation in average temperature between day time and night. It receives high and reliable annual rain fall of about 1375mm ranging from 1350-1400mm. The long dry season lasts from December to May. The area experiences a mean annual temperature of about 32 ^{0}C. The high average monthly temperature occurs in May that ranges between 29 ^{0}C to 32 ^{0}C. The coldest month is August when the average monthly minimum temperature is 21 ^{0}C (National Meteorological Services Agency, 2016). The livestock population of the district is 44,306 cattle, 12,542 goats, 3,558 sheep, 6,172 equines (donkey and mules) and 44,002 poultry and livelihood of the society largely depend on mixed livestock and crop production (Asossa Zone Office of Agriculture, 2018).

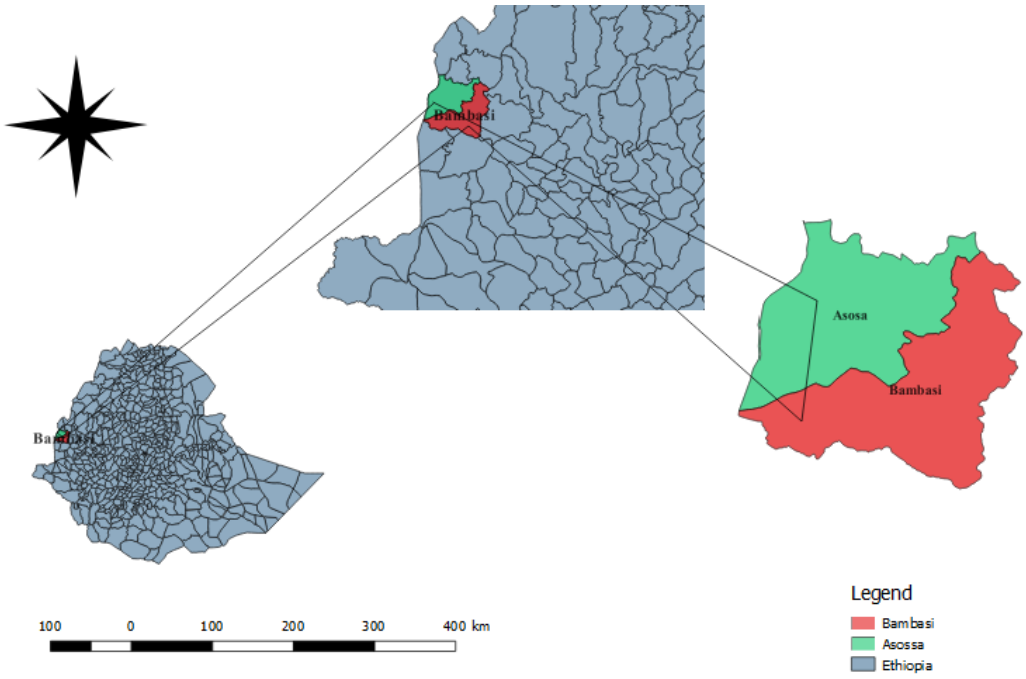


Figure 5: Geographical location of the study area

The study area map was created using Q GIS software as indicated above.

3.2. Study animals

The study animals were sheep and goats of all age, sex and breed which were kept by small holder farmers and showing clinical signs of the disease.

3.3. Study design

Cross-sectional study design (Thrusfield, 2005) was conducted from November 2019 up to April 2020 in Asossa zone, Benishangul-Gumuz Regional State. Active outbreaks were assessed by frequent observation and communicating with zonal, regional laboratory and district animal health professionals who were working in the district veterinary clinic. Field investigation was carried out to identify the clinical signs of the diseases, estimate mortality, morbidity and case fatality rate. Samples were purposively collected from both Asossa and Bambasi districts of Asossa zone at specific sites of

outbreaks from all sheep and goats showing clear signs and/or suspected signs of PPR diseases for virus isolation and molecular detection.

3.4. Sample collection and procedures

3.4.1. Field investigation, sample collection and transportation

A total of 27 (22 nasal swabs [13 from sheep and 9 from goat] and 5 rectal swabs [3 from sheep and 2 from goat/s]) swab sample were purposively collected from sheep and goats showing suspected signs of PPR disease in Asossa and Bambasi districts of Asossa zone at the specific sites/villages where outbreaks were occurred. The samples were collected according to the procedure of OIE manual (2019) for virus isolation and molecular characterization based on clinical evidence of the disease in sheep and goats during early stages of the disease outbreaks.

Collected swab samples were placed in to falcon tubes containing 2ml of virus transport medium (VTM, pH 7.2-7.6) containing phosphate buffer solution (PBS) that contains antibiotics, antifungal agents and phenol red) were added to each sample. The sample tubes were labeled with species of animal, identification number (sample code), sample collection site (village), sample type and date. Moreover the sex, age, vaccination status, agro ecology, body condition, source of animals and others useful data were recorded. The collected samples were immediately placed in the ice box and transported to Asossa regional animal health diagnostic center and kept at -20 ^{0}C for one month until it was transported for analysis at National Animal Health Diagnostic Center (NAHDIC).

3.4.2. Penside (field) test for PPRV Ag detection

During outbreak investigation, commercial lateral flow diagnostic test for PPRV antigen detection (Baron *et al*., 2014) was used in the field for the detection of PPRV responsible for outbreak from animals showing suspected signs of the disease. The test was based on lateral flow technology conducted by taking nasal swabs from suspected animals, then the swabs were rinsed with 10 drops of buffer 19 in cryovial tubes and the suspension

was applied to one end of a chromatographic strip drop by drop to allow the flow of the sample through the strip. Then, the samples were mixed with colored beads coated with a specific monoclonal antibody (Mab) that recognizes PPRV antigen. The sample that contains PPRV antigen were captured by or binds to the beads forming the antigen-bead complex which later on captured by a line of anti-PPRV MAb part-way or when it moves along the chromatographic strip, making a colored line to indicate a positive result. In the absence of PPRV antigen, no beads were bound by the test line and no colored line is formed except positive control line (Baron *et al.*, 2014).

3.5. Laboratory technique

3.5.1. Cell culture and Virus isolation

Nasal swab samples collected in virus transport media (VTM) was initially freeze-thawed thrice and used directly for isolation. All nasal swab samples were homogenized and centrifuged at 3000 rpm for 20 minutes at 4 ^{0}C and supernatant were used for virus culture as indicated by Gomes *et al.* (2016). 100 microliters (µl) of supernatant sample were used as an inoculum to infect (>70% confluence) Vero Dog SLAM cells (VDS) grown on 24 well plate of size 2 cm^2 by preadsorption method. The inoculated cells were incubated at 37 ^{0}C, 5% CO_2 and 96% humidity adjusted incubator for one hour with slight intermittent shaking at 15 minutes intervals to allow adsorption of the virus. The virus inoculum was decanted that was followed by washing of infected cells with Dulbecco's Minimum Essential Medium (serum free DMEM) to which 500 µl of maintenance medium (DMEM with 2% serum) were added. The inoculated cells were observed under inverted electro-microscope for any non-specific reactions and incubated at 37 ^{0}C and 5% CO_2 for seven days. The cells were then monitored daily under inverted electro-microscope for cytopathic effects due to viral replication. The samples were processed up to 3rd passage to declare that it was negative for PPR virus (Mallinath *et al*., 2018).

3.5.2. Immuno-capture ELISA (Antigen capture ELISA)

PPR antigen capture ELISA (FAO/OIE reference laboratory, CIRAD, Montpellier, France) was used to test for the presence of the virus in the samples. Accordingly all the samples were first vortexed for proper homogenization and 25 µl of samples were dispensed to the plates per wells coated with an anti-nucleoprotein (N) capture antibody. 25µl of dilution buffer (ID.vet, France) were added to each wells, two negative control wells (A1 & B1) and positive control wells (C1 & D1). The plates were agitated at room temperature (26 ^{0}C) for two minutes and sealed with adhesive plastic sealants to avoid evaporation of the sample during incubation. Then the plates were incubated at 37 ^{0}C for 45 minutes to allow the antigen antibody complex to be formed. Then the plates were washed six times with 300µl wash solution (ID.vet, France). A 100µl of 1X conjugate (MAb-Horse radish peroxidase, ID.vet, France) were added to each well and incubate at room temperature for 30 minutes. Again each plate wells were washed six times with 300µl wash solution to remove any unbound, antigen, conjugates or antibodies. Finally, 100µl of substrate solution (TMB, ID.vet, France) was added per well per plate that was incubated for 15 minutes at room temperature in the dark for color development. Finally 100µl stop solution (sulfuric acid, ID.vet, France) was added to each well to stop the reaction and optical density (OD) reading were recorded at 450nm using ELx800 BioTek ELISA reader (BioTek, USA). The OD values of each sample were converted to percentage positivity by using the following formula:

S/P % = [OD samples–OD NC/OD PC– OD NC] X 100

The samples with S/P greater than or equal 20 % were considered as positive.

3.5.3. Sample processing and RNA extraction

The collected swab samples in virus transport media (VTM) were squeezed into new tubes. The suspensions were clarified by low-speed centrifugation at 3000 rpm for 20 min at +4 ^{0}C and the supernatants were collected in sterile tubes. RNA extraction was done using the Qiagen RNeasy mini kit (Hilden, Germany) by distributing 560 µl of lysis buffer (AV1) in to all micro centrifuge tubes prepared as per the number of samples. A 140 µl of sample was added into the tubes while RNA free water was added to control tubes with vortexing of the mix. The samples were then incubated at room temperature for 10 minutes and mixed by briefly centrifuging at 8000 rpm for 15 seconds that was followed by adding 560 µl of absolute alcohol to each with subsequent vortexing and brief centrifugation. A total of 630 µl of the solution was added to QIAamp mini column and centrifuged at 8000 rpm for one minute in the same way to perform for the remaining solutions or left over. Add 500 µl of wash one (AW1) solution and centrifuged at 8000 rpm for 1 minute. Any liquid passing through the column will be discarded that is followed by adding 500 µl of wash two (AW2) buffer and centrifuged at 14000 rpm for 1 minute in similar way. The centrifugation was repeated without addition of any reagent for 1 minute. Then, the column was transferred to new collection tube to which 60 µl of elution buffer was added, incubated at room temperature for two minutes and centrifuged at 8000rpm for 1 minute. Then, the extracted RNA was stored at -80°C until analysis is done by RT-PCR.

3.5.4. Real time RT-PCR

The collected samples were examined for the presence of PPRV RNA by real time reverse transcription PCR targeting N-gene of PPRV using QIAGEN® one step real time RT-PCR kit. The reverse transcription and PCR was carried out sequentially in the same tube. The RNA obtained was then converted to cDNA using a reverse transcriptase enzyme. The cDNA were amplified using PPRV specific NP3 forward primer (5′-AGAGTTCAATATGTTCTTAGCCTCCAT-3′) and NP4 reverse primer (5′-TTCCCCACTCACTCTVCTTTGT-3′). The master mix contained the following reagents: 2.6µl of RNase-free water, 10µl of express superscript of PCR supermax

(universal), 0.4µl of ROX (at 1 to 10 dilution), 2µl of express superscript mix, 1.6µl of each primer; NP3, NP4, and 0.4µl PPR probe: FAM-CACCGGAYACKGCAGCTGACTCAGAA-QSY (TAMRA) where a total of 510µl of PCR mix prepared for each and 17µl of the mix were added to each samples (OIE, 2019).

The amplification were carried out with the final reaction volume of 20µl containing 17µl of the prepared master mix and 3µl of RNA template. This mixture were submitted to a thermal cycler for cDNA synthesis at 50 ^{0}C for 15 minutes, denaturation of reverse transcriptase at 95 ^{0}C for 20 seconds, denaturation at 95 ^{0}C for 3 seconds followed by annealing at 60 ^{0}C for 30 seconds and final extension at 60 ^{0}C for 30 seconds in applied biosystem 7500 fast real time PCR system machine (Singapore) for 45 cycles. Threshold cycle (Ct) for each sample was calculated accordingly where the samples with Ct value <35 (cut off value) were considered positive for PPR virus (Kwiatek *et al*., 2010; Munir *et al*., 2013d).

3.5.5. Conventional RT-PCR

Initially RNA was extracted from nasal swab sample suspensions using Qiagen RNeasy min kit (Hilden, Germany) and amplification was done by one step RT-PCR as described above with the primer pair (NP3: 5'-GTC TCG GAA ATC GCC TCA CAG ACT-3' and NP4: 5'-CCT CCT CCT GGT CCT CCA GAA TCT-3') that was used to amplify 351 bp fragment of N gene of PPRV as described by Couacy-Hymann *et al*. (2002). Briefly, the amplification reaction was carried out in a volume of 25 µL containing 7.5 µL RNase free Water, 5 µL 5X PCR buffer, 1 µL dNTPs, 5 µL Q-solution, 1.5 µL NP3(Forward primer) and NP4 (reverse primer) each, 1 µL enzyme mix and 2.5 µL RNA template. The following thermal profile was followed for both reactions: reverse transcription at 50 ^{0}C for 30 min, initial denaturation at 95 ^{0}C for 15 min, and then 40 cycles of denaturation at 94 ^{0}C for 30 seconds, annealing at 60 ^{0}C for 30 seconds and elongation at 72 ^{0}C for 1 minute, followed by a final elongation at 72 ^{0}C for 5 min in a Biometra GmbH flex cycler^2 PCR machine. The PCR product was visualized by runing10 µl of the amplified PCR products on gel electrophoresis at 120V/100mA for 45 minutes. 1.5% agarose gel

(hardy diagnostics, USA) was prepared in Tris borate ETDA buffer (Invitrogen, UK) for running the reaction and the amplicon was stained with SYBR Safe DNA gel stain (Invitrogen, USA). Finally the PCR product or the DNA bands were visualized under Molecular imager gel DOC^{TM} XR imaging system with image lab^{TM} software visualized under UV transilluminator (UVETIC, UK).

3.6. Ethical clearance

Ethical clearance for this study was obtained from animal research ethical review committee of Addis Ababa University College of Veterinary Medicine and Agriculture for collecting samples from the peste des petits ruminants disease suspected sheep and goats as indicated below in Appendix 2 with approved certificate reference number of VM/ERC/21/02/12/2020. All activities done on animal were conducted in accordance to animal research ethics.

3.7. Data management and analysis

All the data generated from the study areas were arranged, checked, coded and entered to Excel spread sheet (Microsoft® Office Excel, 2010). Simple descriptive statistical analysis will be done using STATA version 12. Percentage was used to calculate morbidity, mortality and case fatality due to the outbreak. Laboratory results were also expressed in percentage to calculate and compare the number of positive samples between each test.

4. RESULTS

4.1. Field investigation and clinical signs

Field investigation of PPR outbreak revealed that the disease occurred in the two districts of the region where both sheep and goats were affected and a total of eight animals (6 goat and 2 sheep) was died of disease. During the study period, the outbreak encountered were limited to some specific location of study areas with limited transmission between herds due to vaccination of most of the sheep and goat in the area that enable the animals to have immunity against the disease. The disease was observed in sheep and goats that did not have vaccination history and in those newly introduced small ruminants from some other areas with unknown history of vaccination. The investigation showed that most of the sampled animals were having clinical signs of nasal discharge (sero-mucoid), fever (39.5 ^{0}C-41 ^{0}C), depression, erosive lesion on the muzzle and lips, diarrhea, coughing, and death (Figure 6), but some of the suspected cases included in the sampling were showing limited signs of the disease as summarized in Appendix 1. The mortality, morbidity and case fatality rate of the disease was higher in goat as compared with sheep (Table 1).

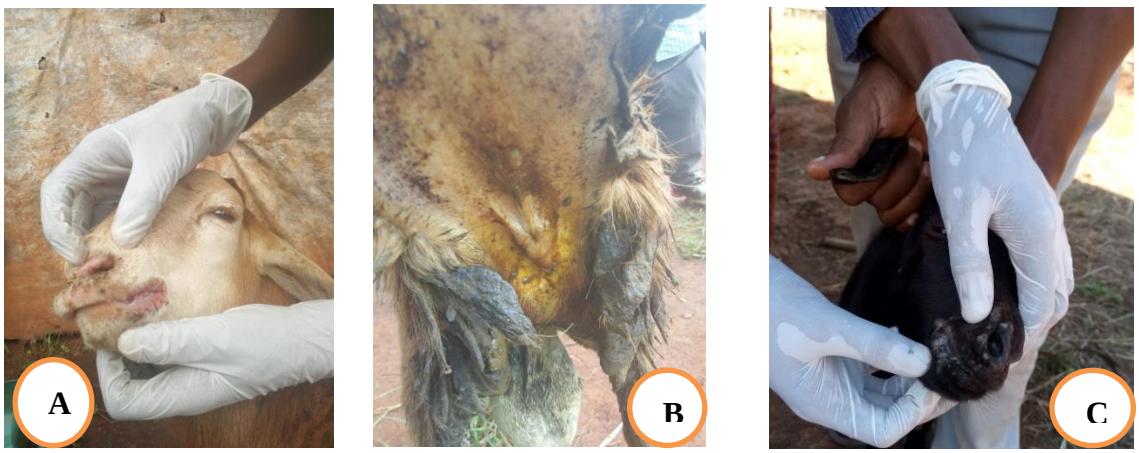


Figure 6: Clinical signs of PPR in sheep and goat

(A)Shows erosive lesions on the lips of sheep, (B) show that the sheep were diarrheic; (C) shows erosive lesions on the muzzle of goat, (D) indicate the nasal discharge due to the diseases in sheep and (E) show goat dead due to the disease.

Table 1: Morbidity, Mortality and Case fatality rate of PPRV

District	species	Total no- of animal in herd at risk	Total No- of infected animals	No- of dead animal	MoR	MR	CFR
Asossa	Ovine	86	15	2	15/86 (17.4%)	2/86 (2.3%)	2/15 (13.3%)
	caprine	60	9	5	9/60 (15%)	5/60 (8.3%)	5/9 (55.56%)
Bambasi	caprine	73	6	1	6/73 (8.2%)	1/73 (1.4%)	1/6 (16.7%)
Overall					30/219 (13.7%)	8/219 (3.7%)	8/30 (26%)

Note that:- MoR=Morbidity Rate, MR=Mortality Rate, CFR= Case Fatality Rate

4.2. Penside test for PPR virus detection

During field investigation of the disease, one to two sheep and/or goats that shows suspected signs of the disease were tested with field PPRV Ag detection penside test kit from each outbreak. Animals with similar clinical signs or with suspected clinical signs of PPR disease were included in sampling considering that there was possibility of the animal to animal transmission in the herd. Accordingly from each five outbreaks sites or

villages, two sheep and/or goats were tested by penside test and thus eight of the tested sheep and/or goats were positive for the test as shown in Appendix 1. Those samples taken from clinically affected sheep and/or goats showed positive for the test as compared with the positive control (Figure 7).

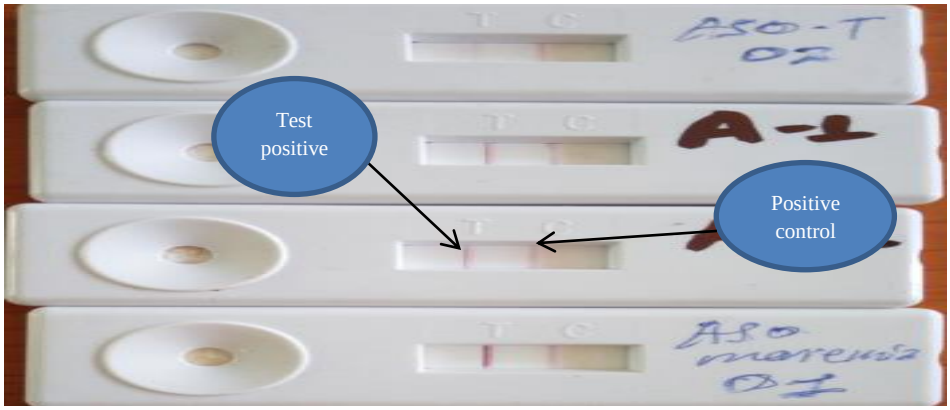


Figure 7: Penside test for detection of PPRV from samples

The above figure showed that PPRV Ag detection penside test kit where the label (C) indicate Positive control and (T) indicate test line that form red line in the presence of PPRV antigen but remain clean (no line) in the absence of the virus.

4.3. Virus isolation on cell culture

All nasal swab samples were inoculated on to Vero Dog SLAM cells (VDS cells) for isolation and propagation of the virus. From these 2/22(9.1%) of the samples showed cytopathic effect from second to fourth day of first inoculation with characteristic CPE of rounding of cells, foamy formation, vacoulation, aggregation and syncytia formation on cells (Figure 8) where the supernatant of the isolate was confirmed using real time RT-PCR and Ic-ELISA. However, the remaining samples were not shown CPE on subsequent observation for seven days. These samples which failed to show CPE were allowed for blind passage until third passage to declare that they were negative for virus isolation. Accordingly, none of the remaining samples showed cytophatic effect in all blind passages; thus considered as negative for the test.

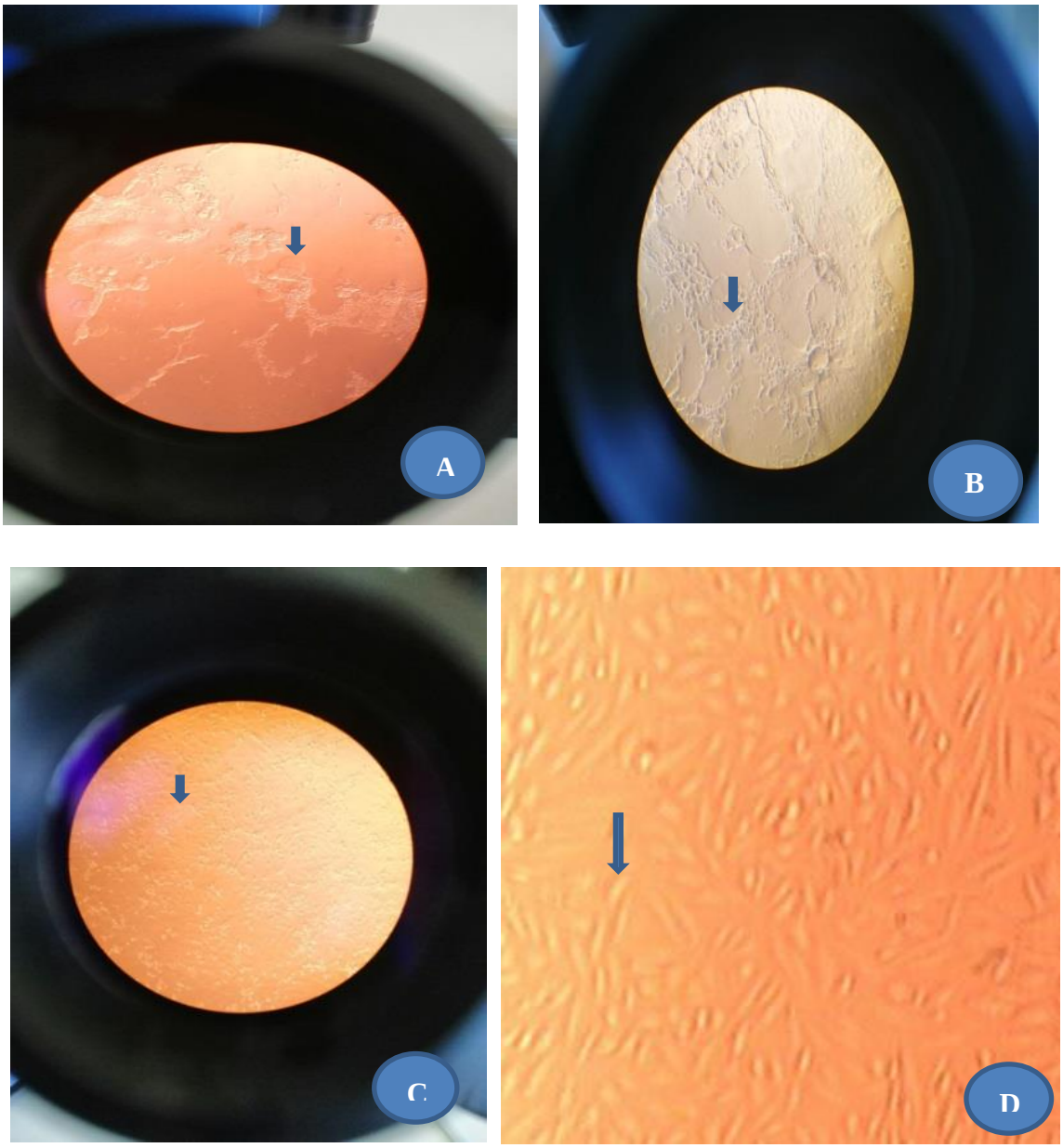


Figure 8: Cytopathtic effect of PPRV on the VDS cells

The arrow head of (A) shows the vacoulation and syncytia formation, (B) foamy formation and (C) rounding of cells and (D) VDS cells with spindle shaped cells (uninfected)

4.4. Immune capture ELISA

Out of the 22 nasal swab sample tested for PPRV Ag using monoclonal antibody coated plate wells by immune capture ELISA targeting the N gene of the PPRV 8/22(36.4%) of the sample were positive for PPRV antigen with the S/P% value above 20% as shown below in Table 2.

Table 2: Summary of laboratory test

District	Species	Sample type	Total no-of sample	IC-ELISA	Real time RT-PCR	RT-PCR	Virus isolation
Asossa	Ovine	NS	13	5/22(22.7%)	6/22(27.3%)	6/22(27.3%)	0/22(0%)
		RS	3	NT	0/3 (0%)	NT	NT
	Caprine	NS	3	1/22(4.5%)	1/22(4.5%)	1/22(4.5%)	0/22(0%)
		RS	1	NT	0/1 (0%)	NT	NT
Bambasi	Caprine	NS	6	2/22 (9.1%)	3/22(13.6%)	3/22(13.6%)	2/22(9.1%)
		RS	2	NT	0/2 (0%)	NT	NT
Total			22	36.3%	45.4%	45.4%	9.1%

Where: NT= Not Tested; NS=Nasal Swab; IC-ELISA=Immunocapture Enzyme Linked Immunosorbent Assay; RT-PCR=Reverse Transcriptase Polymerase Chain Reaction

4.5. Real time RT PCR

Real time RT-PCR detection of all clinical samples showed that 10/22 (45.4%) were positive for PPRV. Of these, 27.3% were sheep and 18.1% were goats (Table 2). Samples that were positive for the test showed Ct value ranges between 17.08 and 30.78 with cut off value <35 were considered as positive (Figure 9).

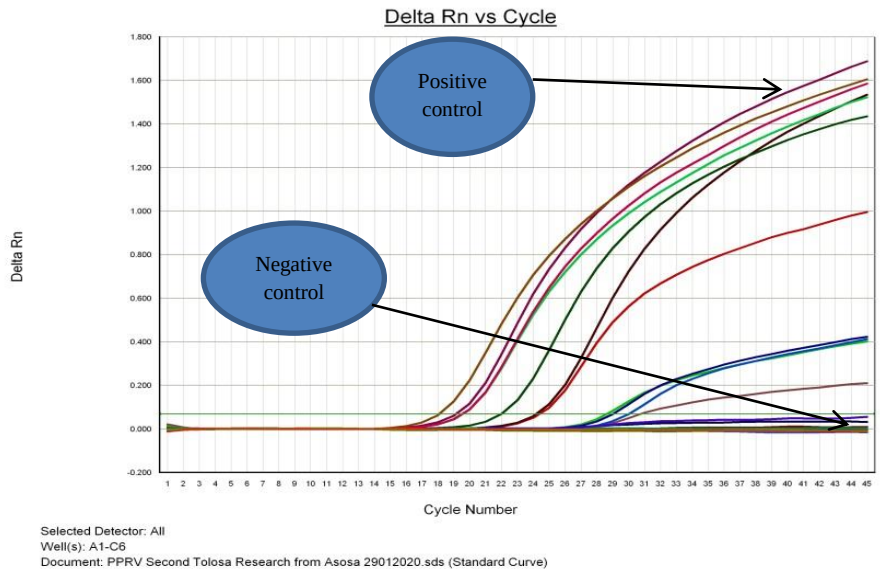


Figure 9: Detection of PPRV using real time RT-PCR

On the above figure, the Real time RT-PCR graph starts rising up from Ct value between 17.06 to 30.78 ranges considered as positive with respect to the positive control whereas, sample with Ct value more than 35 showed no rise in the graph and considered as negative as compared to negative control.

4.6. Conventional RT-PCR

From the 22 nasal swab samples tested by using RT-PCR targeting the N gene of the virus 10/22(45.4%) of samples were positive for the PPRV as indicated in the gel electrophoresis of the PCR products with the fragment size of the amplified products of around 351 bp as reported by Couacy-Hymann *et al*. (2002). Samples with amplified fragment size of approximately 351 bp were considered as positive samples for the test as compared to positive control (Figure 10).

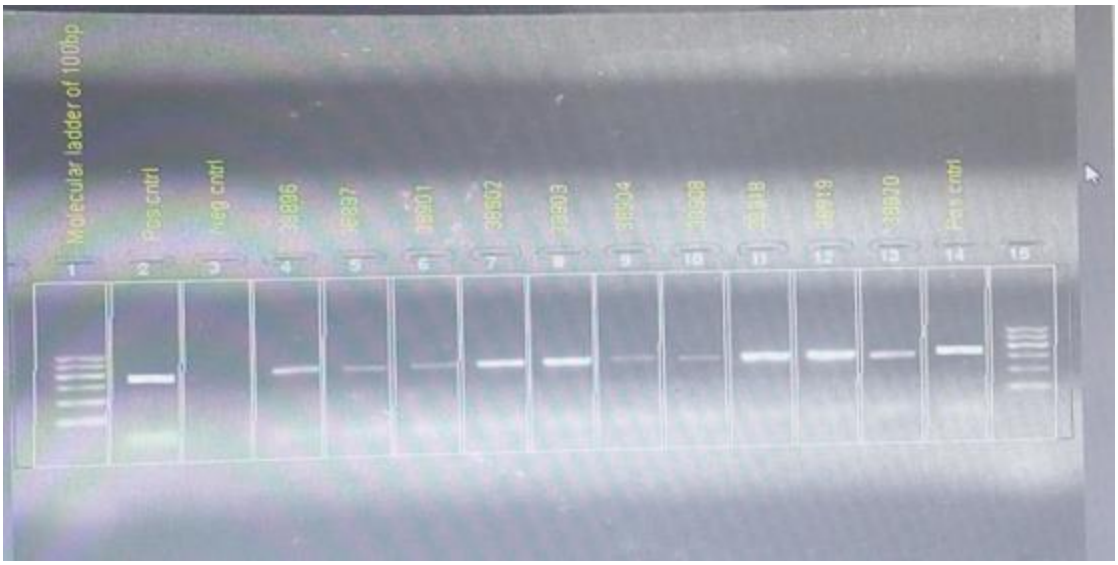


Figure 10: Conventional RT-PCR showing 351 bp of N gene of PPRV

As indicated above the arrow head, show the N gene of PPRV amplified fragment size for the positive control that lies between 300-400 bp of the molecular ladders where the negative control have no any band. Samples that show similar band size with the positive control were considered as positive for the PPRV with approximately fragment size of 351bp.

5. DISCUSSION

This finding revealed that PPRV has been circulating in two districts of Asossa zone as evidenced based on clinical finding, virus isolation and molecular detection indicating that the virus is responsible for the outbreak of the disease that result in production losses and death of sheep and goats. *Peste des petits ruminants virus* was circulating in different parts of Ethiopia causing a lot of production loss and mortality as reported by Roeder *et al*. (1994); Abraham *et al*. (2005); Waret-Szkuta *et al*. (2008); Megersa *et al*. (2011); Alemu *et al*. (2019) and Rume *et al*. (2019).

The clinical finding observed during field investigation of the disease from suspected small ruminants showed that the etiology of the outbreak was peste des petits ruminants' virus. The clinical feature of PPR diseases observed in the sheep and goats in the districts were similar to the clinical signs/manifestations described by Munir *et al*. (2013c); Kgotlele *et al*. (2014); Soltan and Abd-Eldaim (2014); Kardjadj *et al*. (2015); Abubakar *et al*. (2016); Rahman *et al*. (2016); Alemu *et al*. (2019) and Mishra *et al*. (2020) who reported fever, dullness, depression, anorexia, mucoprulent ocular and nasal discharge, erosive stomatitis or lesions on gums, diarrhea, coughing and in sever case death of the animals.

The severity of disease was different between sheep and goat where mortality, morbidity and case fatality rate was higher in goat as compared to sheep. This finding is in concurrent with previous studies that reported the severity of peste des petits ruminants disease were more sever in goats than sheep by El-Rahim *et al*. (2010); Abubakar *et al*. (2011, 2018); Soltan and Abd- Eldaim (2014) and Elhaig *et al*. (2018). This may be because PPRV can cause marked immune suppression in infected goats than sheep (Rajak *et al*., 2005), cause inhibition of leukocyte proliferation (Heaney *et al*., 2002) and Apoptosis of peripheral blood mononuclear cells (Mondal *et al*., 2001). Moreover, it may be associated with virulence strain of the virus, health status or physiological status of the animal, concurrent parasitic and bacterial infections that are likely to worsen the diseases

because of the immunosuppressive effect of the PPRV (Emikpe *et al*., 2010; Malik *et al*., 2011). However, some authors reported that sheep and goats were affected with equally overwhelming consequences with high morbidity and mortality (Taylor *et al*., 2002). This may be because of virulence difference in different PPRV strains (Couacy-Hymann *et al*., 2007a); host species and breed susceptibility (Diop *et al*., 2005) and host adaption to the disease.

The overall morbidity, mortality and case fatality rate were 13.7%, 3.7% and 26% among sheep and goats, respectively. This finding was in accordance with Kardjadj *et al*. (2015) who reported an overall morbidity, cumulative mortality and case fatality rates among sheep and goats of 12.2%, 2.5%, and 20.3%, respectively and the finding of Muthuchelvan *et al*. (2014) who reported morbidity, mortality and case fatality rates of the disease as 16.67%, 2.78% and 16.67%, respectively. These findings are in complete concurrence with Diallo (2006) who reported that morbidity and mortality rates due to PPR may vary from 0 to 90% depending on the local husbandry practices, breed, age and other factors. Report by Balamurugan *et al*. (2012) support the current finding that, mortality in susceptible flocks varies from 10 to 100% and even lower than 10% and morbidity ranges from 50 to 100% where this difference may be due to intensive vaccination program implemented for the target species. Moreover, Abu Elzein *et al*. (1990) also reported the mortality of 50-90 per cent and sometimes nil, and morbidity of 10 to 100 percent, and even lower than 10 per cent indicating that the difference is depending on circumstances.

For confirmation of the cases samples collected based on observed clinical sign of PPR diseases were tested by penside Ag detection test in the field and Ic ELISA for detection of viral antigens in all suspected samples collected from both districts where the tests has the same sensitivity and specificity as described by OIE (2019). The result from the current study showed that 8/22 (36.4%) of the sample were positive for the tests. These indicate that pest des petits ruminants virus is responsible for the diseases outbreak and circulating in herd in both districts of Asossa zone.

The current finding is in agreement with the results reported by Abubakar *et al*. (2011) who reported 34.3% positivity by Ic ELISA and Chauhan *et al*. (2014) who detected 37.4% (49/131) positive results from nasal swabs tested by S-ELISA. But, it is much higher than the finding reported by Munir *et al.* (2009, 2012b) and Alemu *et al*. (2019) who reports 21.4%, and 25.7% and 31.3% respectively. This may be due to proper diagnosis of the diseases in the field that enhance the chance of high positivity. The current finding is lower than the finding reported by Saeed *et al*. (2009), Abubakar *et al*. (2008, 2015b), and El-Rahim *et al*. (2010) who reported 42.6%, 40.98%; 70% and 75% positivity using immune capture ELISA respectively. This may be because of the sample type, number of animals included, proper diagnosis or observation at field, sample collection and storage for long time.

Moreover, the detection ability of Ic ELISA was lesser than RT-PCR. This is attributed to high sensitivity and specificity of RT-PCR as compared with Ic ELISA as described by OIE (2019) and report of Khan *et al*. (2013) who indicated that detection ability of Ic ELISA was dependent on the concentration of the virus in the given sample (sample type that contain high concentration of virus) where lymph node has high concentration of virus than swabs (nasal, ocular and rectal) that showed high percentage of positivity by RT-PCR than Ic ELISA.

From all samples analyzed using conventional RT-PCR and real time RT-PCR, 45.4% were positive for PPRV from nasal swabs but none of the rectal swabs were positive for PPRV. This could be because of less frequent detection of the virus in faecal material as PPRV was detected later post-infection in fecal material than in other body fluids and the detection was intermittent as reported by Parida *et al*. (2019). The current finding is in line with the finding reported by Sait and Dagalp (2019), Alemu *et al*. (2019) and Chauhan *et al*. (2014) who reported 45.8%, 46.4% and 48% positivity from swab samples by RT-PCR, respectively. It is also agreed with Kwiatek *et al*. (2011) who reported 44.4% positivity in morocco using RT-PCR but, higher than the report by Anees *et al*. (2013) and De Nardi *et al*. (2012) who found 25% (8/32) and 33.3% (7/21) positive samples by the amplification of the nucleoprotein (N) gene and F gene, respectively. This

may be because of level of positivity influenced by the sample type used during diagnosis of PPR, stage of infection, and the type of gene targeted for RT-PCR (Luka *et al*., 2012). Moreover, this finding is lower than the report of Luka *et al*. (2011), Kardjadj *et al*. (2015) and Kabir *et al*. (2020) who detected 51.2% (17/33), 58.06% and 78.95% positivity by RT-PCR, respectively. The differences in positivity rate may be related to the detection method, sample size and the presence of other exacerbating diseases such as pasteurellosis, caprine contagions pleuropneumonia (CCPP), bluetongue (BTV), contagious ecthyma and foot and mouth disease (FMD) which can occur concurrently (Couacy-Hymann *et al*., 2005).

The expected fragment size obtained during this study by amplifying N-gene of the virus using conventional RT-PCR was approximately 351 bp. This finding is in agreement with the finding of Ularamu *et al*. (2012) and Soltan and Abd-Eldaim (2014); Couacy-Hymann *et al*. (2002); Kardjadi *et al*. (2015); Rahman *et al*. (2016) and Nabi *et al*. (2018) who reported the fragment size of 350, 351 and 352 bp respectively based on amplification using primers targeting N-gene of PPRV.

All nasal swab samples were allowed to grow on VDS cells for isolation and propagation of PPR virus. Thus from all samples only two samples were grown on their first inoculation with characteristic CPE of PPRV such as rounding, vacuolation, foamy formation and aggregation or fusion of cells to form syncytia as described by OIE (2013); OIE (2019) and Adombi *et al*. (2011). The presence of the virus was confirmed by testing the supernatant of isolated virus by Immunocapture ELISA and real time RT-PCR. This finding is also in agreement with the finding of Hegde *et al*. (2009); Rahman *et al*. (2011), Zahur *et al*. (2014) and Elsheikh *et al*. (2019) who reported similar CPE on vero cells showing cell rounding, vacuolation and formation of small syncytia. Gomes *et al*. (2016) and Nafea *et al*. (2019) also isolated the virus on Vero cell that showed rounding, aggregation and syncytia of cells as characteristic CPE of the virus. In addition, as reported by Sannat *et al*. (2014) PPRV showed rounding, ballooning of cells and later on aggregation of cells followed by formation of fusion mass and syncytia on Vero cell and Vero/SLAM cells.

In the current study, PPRV was detected in 9% (2/22) of clinical samples by virus isolation. This finding were consistent with the finding reported by Zahur *et al*. (2014) who reported 9.3% (4/43) of samples positive for virus isolation inoculated on vero cells. However, this finding is lower than the finding reported by Elsheikh *et al*. (2019) and Elhaig *et al*. (2018) who detected 70%, 83% positivity or growth of PPRV on Vero cells respectively. The difference observed could be due to low sample quality, low tenacity of PPRV, and problem associated with transportation and storage of samples at -20 ^{0}C for long duration.

6. CONCLUSION AND RECOMMENDATIONS

In this study, the presence of PPR in small ruminants of Asossa zone was confirmed by two different tests namely IC-ELISA and Molecular methods (RT-PCR and real time RT-PCR). The disease was detected in non-vaccinated herds but not in vaccinated animals which are believed to be immune to the disease. The mass vaccination carried out in the region showed improvement in control and prevention of the disease in the region. However, as evidenced from virus isolation and molecular detection of PPRV indicated, the virus was circulating in the area causing outbreak at different time throughout the year where all of the cases were associated with unvaccinated and newly introduced sheep and goats from neighboring region and districts of the region. Thus, the disease is still remain as a challenge to small ruminant health as it causes mortality, production and productivity loss in the study area. This indicates the possibility of the virus spread to different disease free districts in the region targeting non vaccinated and young group of sheep and goats posing further loss in production and mortality. Therefore, based on the above conclusive evidence the following recommendations are forwarded:

- ✓ Further investigation should be done on the epidemiology and genetic analysis of the virus to the lineage level via generation of gene sequence data and molecular evolutionary relationship analysis.
- ✓ Vaccine coverage and strategies should be improved and implemented including the newly introduced sheep and goat.

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8. ANNEXES

Annex I: Standard operation procedure for subculture of monolayer cells

Scope

The scope of this procedure is applicable to subculture monolayer cells in cell culture laboratory

Principle

When the cell density reaches a level such that all of the available substrate is occupied or when the cell concentration exceed the capacity of the medium, growth ceases or is greatly reduced. At this time the culture must be divided.

Materials and reagents

- Cells to be sub cultured
- Growth medium
- Trypsin EDTA (mostly 0.25%)
- PBS 1X
- Ethanol 70% in spray bottle
- Pipette of different volume
- Pipette cylinder containing disinfectants
- Bio safety cabinet
- Pipette aid
- Refrigerator
- Tissue paper (wipes)
- CO_2 incubator
- Permanent marker
- Centrifuge tube 50ml
- Tissue culture flask
- Water bath
- Note book

Procedures

- Prepare the hood and bring the reagents and materials to the hood to begin the procedure. Trypsin and PBS should be pre warmed at 37 ^{0}C in water bath
- Examine the culture carefully for signs of deterioration or contamination
- Take the culture flask to sterile work area (bio safety cabinet) and remove and discard the medium. Handle each cell line separately, repeating this procedure from this step for each cell line handled.
- Add PBS (0.2ml/cm^2) to the side of the flask opposite the cells so as to avoid dislodging cells, rinse the prewash over the cells and discard. This step is designed to remove trace of serum that would inhibit the action of trypsin and deplete the divalent cations necessary for cell adhesion.
- Add trypsin (0.1ml/cm^2) to the side of the flask opposite to the cells. Turn the flask over and lay them down. Ensure that the monolayer is completely covered and leave the flasks stationary for 15-30 seconds.
- Raise the flasks to remove the trypsin from the monolayer and quickly check that the monolayer is not detached. Using trypsin at 4 ^{0}C helps to prevent premature detachment, if this turn out to be problem.
- Withdraw all but a few drops (0.04ml/cm^2) of the trypsin
- Incubate with flask lying flat until the cell round up; when the bottle is tilted the monolayer should slide down the surface (this usually occurs after 5-15 min). do not leave the flasks longer than necessary, but on the other hand, do not force the cells to detach before they are ready to do or else clumping may result.
- Add medium (0.1-0.2ml/cm^2) and dispense the cells by repeated pipetting over the surface bearing the monolayer.
- Finally, pipette the cell suspension up and down a few times, with the tip of the pipette resting on the bottom corner of bottle, taking care not to create foam. The degree of pipetting required will vary from one cell line to another. A single cell suspension is desirable at subculture to ensure an accurate cell count and uniform growth on reseeding.
- Count the cells

- Dilute the cell suspension to the appropriate seeding concentration and distribute among the flasks
- Incubate in 5% CO_2 at 37 ^{0}C.

Annex II: Procedure for preparation of clinical specimens and inoculation of cell lines

Purpose

To provide a protocol for preparation of clinical specimen and isolation of viruses by inoculation of suitable cell lines which is being used in cell culture laboratory.

Principle

Viruses are obligate intracellular parasites that require living organisms in order to replicate. Culture cells, eggs and laboratory animals may be used for virus isolation. Although embryonated eggs and laboratory animals are very useful for the isolation of certain viruses, cell cultures are the sole system for virus isolation in most laboratories.

Reagents

- A monolayer cell (>70% confulence)
- PBS with antibiotic and antimycotic
- 70% alcohol
- Sterilized distil water
- Growth medium with antibiotic and antimycotic
- 2% virkon's or other disinfectants

Materials

- Scissor
- Forceps
- Stomacher or other tissue grinder (mortar and piston)
- Vortex mixer
- Centrifuge

- ♥ Refrigerator
- ♥ Freezer (-20 to -80 ^{0}C)
- ♥ 15 ml centrifuge tube
- ♥ 5ml tube
- ♥ Biosafety cabinet II
- ♥ Personnel protective equipments
- ♥ 1,2,5 and 10 ml pipette and pipette aid
- ♥ Permanent marker
- ♥ Hand sprayer with 70% ethanol
- ♥ Beaker with 2% virkons or other disinfectants
- ♥ Sterile sand
- ♥ Pipette cylinder containing disinfectants

Procedures

Sample preparation

Swab

- An identical procedure is used for preparation of nasal, throat, rectal and vesicle swabs
- The swab is macerated well in transport medium itself used for collection
- The resulting suspension is transferred to centrifuge tube and centrifuge at 3000 rpm-5000 rpm for 20 minutes
- The supernatant is collected and ready to be inoculated in to suitable cell lines.

Inoculation of suitable monolayer cell with the collected supernatant

- Select monolayer cell culture with a confluence of >70% and remove growth medium
- Wash the culture twice with PBS

- Inoculate the specimen suspension on the test flask and PBS on the negative control flask (0.4ml/cm^2 tissue culture flasks) and incubate at 37 ^{0}C for 60 minute to allow the virus to absorb on to the cell culture
- Add maintenance medium and incubate the flask at 37^{0}C for appropriate time of 3-7days. And keep the control flask without any specimen inoculums.
- Observe for the effect of virus action
- It is essential that each material be passed in cell culture at least three times before declaring any specimen negative. The presence of virus is detected by observing cytophatic effect.

Annex III: Procedures of Immuno-capture ELISA

SCOPE

Immune capture is designed to detect peste des petits virus in different matrices. It uses an anti-nucleoprotein (N) capture antibody and an anti-N monoclonal HRP antibody for revelation. It can be used on live animals or post mortem diagnosis.

The test can be used on:

- ✓ Oral, rectal (dry or containing preservation liquid), ocular and nasal swabs
- ✓ Tissue samples (lung, liver, spleen, heart, kidney, intestine and lymph nodes)
- ✓ Gum debris

Principle

Wells are coated with an anti PPRV-N antibody. Samples to be tested and controls are added to the microwells. PPRV, if present forms an antibody antigen complex. After washing, an anti PPRV-N Mab-HRP conjugate is added, forming an antibody–antigen-Mab-HRP complex. After washing in order to eliminate the excess conjugate, the substrate solution (TMB) is added.

The resulting coloration depends on the quantity of the PPRV present in the samples to be tested.

- In the presence of PPRV, a blue coloration appears which become yellow after addition of the stop solution.
- In the absence of PPRV, no coloration appears

Materials required

- 37 ^{0}C incubator
- Mono or multichannel pipette capacity of delivering volume of 5µl, 10 µl, 100 µl and 200 µl.
- Disposable tips
- 96-well microplate reader
- Distilled or deionized water
- Manual or automatic wash system

Wash solution preparation

If necessary bring the wash concentrate (20X) to room temperature (21 ^{0}C ±5^{0}C) and mix thoroughly to ensure that the wash concentrate is completely solubilized. Prepare the wash concentrate (1X) by diluting the wash concentrate (20X) in distilled water or deionized water.

Positive control preparation and storage

Reconstitute the freeze dried positive control with positive control. Reconsititution solvent is supplied in the kit. The volume to be added is mentioned on the label of each kit. Wait approximately 5 minute and mix gently but thoroughly ensure complete solubilisation.

Once the positive control is reconstituted it should be stored at:

- For 4 weeks at 5 ^{0}C (±3^{0}C)
- For long term storage aliquot and freeze (<-18^{0}C). each aliquot can support 3 freezing thawing cycles without loss of activity and can be stored for 18 months.

Sample preparations

Swabs

- ✓ Elution should be performed in approximately 200µl of dilution buffer 13 and deposit 50 µl of the eluate undiluted in the well (do not add 25 µl of dilution buffer 13 to this sample well)
- ✓ Elution should be performed between 2-4hours at room temperature or preferably overnight at 5 ^{0}C (±3^{0}C) in a wet chamber.
- ✓ Elution in PBS is not recommended because antigen extraction is improved in dilution buffer 13.

Procedure

- ❖ Allow all reagents to come to room temperature (21^{0}C±5^{0}C) before use. Homogenize all reagents by inversion or vortex.
- ❖ Add
  - ✓ 25µl of dilution buffer 13 to all wells.
  - ✓ 25 µl of the negative controls to wells A1 and B1
  - ✓ 25 µl of the positive control to wells C1 and D1
  - ✓ 25 µl of each samples to be tested to the remaining wells
- ❖ Agitate the plate for 2 minute (±1min) at 21 ^{0}C±5^{0}C
- ❖ Cover the plate and incubate 45 min±5min at 37 ^{0}C
- ❖ Wash each well 6 times with approximately 300 µl of the wash solution.
- ❖ Prepare the conjugate 1X by diluting the conjugate 10x to 1/10 in dilution buffer 19
- ❖ Add 100 µl of the conjugate 1x to each well
- ❖ Cover the plate and incubate for 30 minute at room temperature
- ❖ Wash each wells 6 times approximately 300 µl of the wash solution
- ❖ Add 100 µl of the substrate solution to each wells
- ❖ Incubate for 15minute at room temperature
- ❖ Add 100 µl of the stop solution to each well in order to stop the reaction
- ❖ Read and record the O.D at 450nm.

Interpretation

For each sample calculate the S/P% expressing the level of PPRV as a percentage of the positive control as follows:

$S/P\% = (OD_{sample} - OD_{NC} / OD_{PC} - OD_{NC}) X100$

Sample presenting a S/P%

- ✓ Less than 20% are considered as negative (no PPRV in the sample tested)
- ✓ Greater than or equal to 20% are considered positive (PPRV is present in tested sample)

Annex IV: Procedure of Extraction of RNA from swab specimens

1. Vortex swab specimen fluid and transfer 500µl of sample into the microcentrifuge tube labeled with the specimen number.

2. Place 500µl of Qiagen RLT with β-ME into the microcentrifuge tube. Vortex for 15 sec. when processing a large number of specimens the RLT buffer can be mixed with the specimen by pipetting up and down vigorously 4 to 6 times.

3. Pulse spin to eliminate liquid specimen in the lid after vortexing. Add 500µl 70% ETOH and vortex well. Centrifuge lysed swab specimen for 5 min. at 5, 000xg in a microcentrifuge at room temperature.

4. Transfer all of the lysed specimen supernatant to a RNeasy Qiagen column that has been marked to identify the specimen. Centrifuge for 15 sec. at >8,000xg at room temperature. Check to assure the entire specimen has flowed through the column. Repeat until all of specimen has been applied to the column.

5. Add 700µl of RW1 buffer to the RNeasy column and centrifuge for 15 sec. at >8,000xg and place the column in a collection tube (the tube with RW1 flow through may be discarded)

6. Add 500µl RPE buffer to the RNeasy column and centrifuge for 15 sec at >8000xg. Discard flow through from the collection tube.

7. Repeat for a total of 2 washes with RPE buffer discarding flow through from the collection tube. Following the last RPE wash, place the RNeasy column in a new 2ml collection tube.

8. Centrifuge the empty RNeasy column an extra 2 minutes at full speed and discard the collection tube.

9. Place the RNeasy column an elution tube or a 1.5ml micro centrifuge tube that has been marked with the specimen number and pipet 50µl RNase-free H2O into the column. Do not touch the silica-gel membrane with the pipettor tip. Incubate at room temperature for 1 minute. Elute RNA by centrifuging for 1 minute at >10000 rpm. Discard RNeasy column. Store at 4oc until specimen is tested on RT-PCR. RNA should be stored at 4oC for as short of period as possible before testing. If the sample cannot be tested within 24 hours, it should be stored at -20 ^{0}C or colder.

Annex V: Procedure of RT-PCR

1. Thaw all reagents, except reverse transcriptase and taq polymerase and possibly keep them on ice

2. Master mix preparation: the Master Mix preparation must be carried out in ice bath (ice flakes). Before preparing the reaction mix, it is necessary to calculate the correct volume of reagents to be used as in attached protocols. Always prepare mix that will be enough for the number of samples to be tested including positive and negative controls plus one. The extra one will be to compensate the loss during pipetting.

3. Prepare a reaction mix according to the table below.

Conventional PCR QIAGEN One Step RT-PCR kit

Reagent (stock)	**µl X 1 reaction**
RNase free water	7.5
5X PCR buffer	5
dNTPs mix 10mM	1
Q-solution	5
Forward primer NP3 (10 µl)	1.5
Reverse primer NP4 (10mM)	1.5
Qiagen Enzyme mix	1
Total volume	22.5
RNA	2.5
Final volume	25

4. Aliquate 22.5µl of the prepared master mix in to approximately labeled 0.2microcentrifuge tubes in PCR work station for master mix

5. Add 2.5µl of RNA template in sample dispensing PCR work station and transfer them to the thermocycler

6. Place the reaction tubes in the thermal cycler and setup temperature according to the protocol in the table below

Amplification cycle

Steps	1		2			3
Cycles	1X		40X			1X
Temperature	50 ^{0}C	95 ^{0}C	94 ^{0}C	60 ^{0}C	72 ^{0}C	72 0C
Time	30 min	15 min	30 sec	30 sec	1 min	5 min

7. After the amplification is completed takeout the PCR product to run on agarose gel 10 µl of this product is analyzed by electrophoresis on 1.5% of agarose gel

Annex VI: Procedure of Agarose Gel Electrophoresis

1. Prepare an adequate volume of 1xTBE-buffer to prepare the gel and to fill the electrophoresis tank

2. The amount of agarose that is used depends on the size of the tray and the concentration required. For general purpose a 2% gel is used. Gels are typically between 0.5 and 1 cm thick.

Tray	1X TBE buffer	1% agarose	2% agarose
10cmX10cm	50ml	0.5gm	1gm
10cmX15cm	75ml	0.75gm	1.5gm
15cmX15cm	112.5ml	1.125gm	2.25gm

3. Prepare a solution of molten agar as required by adding the 1xTBE-buffers to the appropriate amount of agarose powder in a suitable flask/bottle. Bring to boil in the microwave oven. Screw the cap loose in order to guarantee the pressure balance within the bottle/flask. After the agarose has been allowed to cool down to about 55 ^{0}C, add the required amount of SYBR safe in chemical hood. This prevents warping of the gel apparatus

4. Seal the ends of the edge of gel casting tray with masking tape and pour the gel in to it. Insert the comb and make sure that there are no bubbles trapped underneath the combs and that all bubbles on the surface of the agarose are removed before the gel sets. Stand for 45-60 min to allow the gel to solidify.

5. After the gel has set, remove the tape from the casting tray, place the gel casting tray containing the set gel in the electrophoresis tank. Add sufficient 1xTBE-buffer to cover the gel to a depth of 1mm (or until the wells are just submerged) and withdraw the gel comb, taking care not to tear the sample wells. Make sure no air pockets are trapped within the wells.

6. Apply 2ul of tracking dye to each 10ul of sample and add samples to the individual wells. Take care not to over load and be sure to include appropriate DNA molecular weight markers.

7. Cover with the safety cover and run gel at 110mA (7x10cm tray) or 200mA (15x10cm tray) - typically 1 to 10v/cm of gel. When the bromophenol blue marker is about two thirds from the top, the gel can be stained with SYBR safe.

8. Turn off the power supply when the bromophenol blue has merged a distance judged sufficient for separation of the DNA fragments.

9. The DNA can be visualized on a UV transilluminator and photographed

9. APPENDICES

Appendix I: Overall information gathered regarding animals during the outbreak investigation

District	Villages	Sample type	species	sex	Age	BCS	VS	PST	Clinical signs			
									Nasal discharge	Fever (39.5-41^{0}C)	Lesion on lip/muzzle	diarrhea
Asossa	Kebele-03	NS	Ovine	M	4yr	Good	NV	+	Sero-mucoid	41 ^{0}C	Present	present
	Kebele-03	NS	Ovine	M	4yr	Good	NV	+	Sero-mucoid	41^{0}C	Present	present
	Kebele-03	NS	Ovine	M	3yr	Good	NV	NT	Serous	40.5^{0}C	Present	present
	Kebele-03	RS	ovine	M	3yr	good	NV	NT	serous	40.5^{0}C	present	present
	Kebele-03	NS	Ovine	M	2yr	Good	NV	NT	Serous	40.7^{0}C	Present	present
	Kebele-04	NS	Ovine	M	3yr	Good	NV	+	Sero-mucoid	40.5^{0}C	Present	present
	Kebele-04	NS	Ovine	M	3yr	Good	NV	+	Sero-mucoid	40^{0}C	Present	present
	Kebele-04	RS	ovine	M	2yr	good	NV	NT	serous	40^{0}C	absent	present
	Kebele-04	RS	Ovine	M	4yr	good	NV	NT	serous	40.3^{0}C	absent	present
	Kebele-04	NS	Ovine	M	2yr	Good	NV	NT	Serous	40^{0}C	Absent	present
	Kebele-04	NS	Ovine	M	3yr	Good	NV	NT	Serous	40.5^{0}C	Absent	present
	Kebele-04	NS	Ovine	M	3yr	Good	NV	NT	Serous	40^{0}C	Absent	present
	Kebele-04	NS	Ovine	M	2yr	Good	NV	NT	Sero mucoid	40.2^{0}C	Absent	present
	Kebele-04	NS	Ovine	M	3yr	Poor	NV	NT	Serous	40.6^{0}C	Absent	present
	Kebele-04	NS	Ovine	M	4yr	Good	NV	NT	Serous	40.3^{0}C	Absent	present
	Kushmengal	NS	caprine	M	3yr	Good	NV	+	Sero-mucoid	40.5^{0}C	Absent	present
	Kushmengal	NS	caprine	M	3yr	Good	NV	NT	Sero mucoid	40^{0}C	Absent	present
	Kushmengal	RS	caprine	M	3yr	good	NV	NT	Sero-mucoid	40.5^{0}C	absent	present
	Kushmengal	NS	caprine	M	2yr	Good	NV	-	Serous	40.5^{0}C	Absent	present
	Baro	NS	Ovine	F	3yr	Poor	NV	NT	Sero-mucoid	39.8^{0}C	Absent	present
Bambasi	Nebar keshmando	NS	caprine	M	5m	Good	NV	NT	Sero-mucoid	39.8^{0}C	Absent	present
	Nebar keshmando	NS	caprine	M	5m	Poor	NV	+	Sero-mucoid	39.6^{0}C	Absent	present
	Nebar keshmando	RS	caprine	M	3yr	poor	NV	NT	Sero-mucoid	39.5^{0}C	Present	present
	Mender-53	NS	caprine	M	3yr	Good	NV	+	Sero-mucoid	40.5^{0}C	Present	present
	Mender-53	NS	caprine	M	3yr	Poor	NV	+	Sero-mucoid	39.5^{0}C	Present	present
	Mender-53	NS	caprine	F	3yr	Poor	NV	NT	Serous	39.7^{0}C	Present	present
	Mender-53	NS	caprine	M	5m	Poor	NV	NT	Serous	39.9^{0}C	Present	present

Note that:-BCS=body condition score, F=female, M= male, m=month, NT= not tested, NS=nasal swab, yr= year, VS=Vaccination status, NV=none vaccinated and PST=penside test

Appendix II: Ethical clearance certificate for sample collection from animals