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MOLECULAR DETECTION OF PESTE DES PETITIS RUMINANTS VIRUS IN OUTBREAK CASES IN THE PASTORAL AREAS OF ETHIOPIA

MSc THESIS

BY

ETSEGENET TEKEBA ADUGNA

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH MVSC PROGRAM IN VETERINARY MICROBIOLOGY

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MOLECULAR DETECTION OF PESTE DES PETITIS RUMINANTS VIRUS IN OUTBREAK CASES IN THE PASTORAL AREAS OF 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

Etsegenet Tekeba Adugna

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Bishoftu, Ethiopia

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

Title: Molecular detection of peste des petitis ruminants' virus in outbreak cases in the pastoral areas of Ethiopia

Submitted by: Etsegenet Tekeba _____ _____

Name of Student Signature Date

Approved *for submittal to* thesis assessment committee

1. Dr. Kebede Amenu (DVM, MSc, PhD) _____ _____

Advisor Signature Date

2. Dr. Redeat Belaineh (DVM, MSc) _____ _____

Co- Advisor Signature Date

3. Dr. Gezahegn Mamo (DVM, MSc, PhD) _____ _____

Department chairperson Signature Date

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Animal Production Studies

As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by Etsegenet Tekeba entitled "Molecular detection of peste des petitis ruminants' virus in outbreak cases in the pastoral areas of Ethiopia" and recommend that it be accepted as fulfilling the thesis requirement for the degree of Masters of Science in Veterinary Microbiology.

_____	_____	_____
Chairman (title and name)	Signature	Date
_____	_____	_____
External Examiner (title and name)	Signature	Date
_____	_____	_____
Internal Examiner (title and name)	Signature	Date
1. Dr. Kebede Amenu (DVM, MSc, PhD)	_____	_____
Advisor	Signature	Date
2. Dr. Redeat Belaineh (DVM, MSc)	_____	_____
Co- Advisor	Signature	Date
3. Dr. Gezahegn Mamo (DVM, MSc, PhD)	_____	_____
Department chairperson	Signature	Date

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Name: Etsegenet Tekeba Signature: _____

College of Veterinary Medicine and Agriculture, Bishoftu

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LIST OF ABBREVIATIONS

AGID	Agar Gel Iimmune-Diffusion
BCO	Branch coordination office
CDV	Canine Distemper Virus
CIEP	Counter Immune-Electrophoresis
cDNA	Complementary Deoxyribonucleic Acid
cELISA	Competitive Enzyme Linked Immunosorbent Assay
cDNA	Complementary Deoxyribonucleic acid
CIEP	Counter immuno-electrophoresis
CSA	Central Statistical Agency
CPE	Cytopathic Effect
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
FMV	Feline Morbillivirus
FAO	Food and Agriculture Organization
IcELISA	Immunocapture Enzyme Linked Immunosorbent Assay
GDP	Gross Domestic Product
LFD	Lateral flow device
MAb	monoclonal antibodies
MV	Measles Virus
MV	Morbillivirus
MoA	Ministry of Agriculture
mRNA	Messenger Ribonucleic acid
NAHDIC	National animal health diagnostic center
NVI	National Veterinary Institute
NP	Nucleoprotein
OD	Optical density
OIE	Office International des Epizootics
ORF	Open reading frame
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PDV	Phocine Distemper Virus
PPR	Peste des Petits Ruminants
PPRV	Peste des Petits Ruminants Virus
RNA	Ribonuclic acid
RPV	Rinderpest Virus
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RVL	Regional veterinary laboratories
SLAM	Signaling Lymphocyte Activation Molecule
SNNP	Southern nation and nationality
TBE	Tris-Borate-ETDA buffer
UTR	Untranslated region
VTM	Viral transport medium
VN	Virus Neutralization

ABSTRACT

In this study a molecular detection of Peste des Petitis Ruminants virus was conducted from outbreak cases in the pastoral areas from January 2017 up to May 2019 with the objective of identifying the virus from outbreak cases from lowland areas using molecular techniques. A total of 60 swab samples were collected from nine outbreaks investigated in six regional states of the country. The outbreak investigation was made by the direct information received from regional veterinary laboratories after the confirmation of the outbreaks by pen-side test. From the total (n=21) samples tested using IcELISA, 18 (87%) were positive and from the total (n=60) samples tested for RT-PCR, 8 (13.3%) were found positive, showing great antigen detection rate difference between the two tests. It was also understood that the virus was detected in higher proportion in younger animals (66.6%) and the goat population (77%) than the rest. Additionally, the virus culture yielded a CPE result by growth on vero cells. The study was able to identify that PPR virus is circulating actively in the small ruminant population, special in the lowland areas, which has a great tendency to expand to the highland areas. Further characterization of the virus detected using molecular technique will help in better understanding of the disease and control program implementations for the future.

Key words: *Lowland, Molecular, Outbreak, PPR, Small ruminant*

1. INTRODUCTION

Peste des petits ruminants (PPR) is an acute, highly contagious, notifiable and economically important transboundary viral disease of sheep and goats caused by a virus which belongs to the genus *Morbillivirus* of family *Paramyxoviridae*. Peste des petits ruminants virus (PPRV) has a single serotype with four distinct genetic lineages (I-IV) and closely related to rinderpest virus, which actually was assumed for a long time to be a variant of rinderpest adopted in small ruminants in causing PPR (Agnès *et al.,* 2008). Sheep and goats are the common hosts of PPRV but other species of animals specifically wild small ruminants can be affected which was evident after death of gazelles in a zoo in the Arabian Gulf in the late 1980s (Tewodros and Melese, 2012). This virus was also suspected to have been involved in a respiratory disease, which affected camels in Ethiopia (Abraham *et al*., 2005). PPR is spreading to new countries, affecting and threatening an increasing number of small ruminant and livestock keepers (FAO, 2013). From the late 1970s onwards, sub-Saharan Africa, then the Middle East and Asia experienced severe epidemics; the process of expansion in to new, uninfected territories continued. Now a days, the disease is recognized to be responsible for mortality and morbidity across many countries of the world including Southern Asian countries Near East, Middle East and Arabian Peninsula countries; the disease is also reported in China (Afera *et al*., 2014; Abid *et al.,* 2009). Clinically affected animals have dyspnea, hyperpnea and coughing. There is also a marked serous to mucopurulent nasal and ocular discharge and erosion or ulceration of the pharyngeal epithelium (Thomas *et. al.,* 2006).

When the disease occurs, up to 100% of the animals may be affected by PPR in a flock and the mortality rate may range from 0 to 90% according to age, breed and species of animals involved. When the infection results in overt and acute disease, the most common outcome is death with case fatality rates that may exceed 90% in naïve populations (Albina *et al*., 2013). Because of the dramatic clinical incidence and associated restrictions on animal and product movements, PPR is considered as a disease of major economic impact and has to be notified to the World Animal Health Organization (OIE).

Despite control efforts in a number of countries, PPR has continued to spread across Africa and Asia, placing an increasing burden on the livelihoods of livestock keepers and on veterinary resources in affected countries. The sheep and goat population of the 65 infected countries is estimated to be 1.44 billion based on 2011 FAOSTAT data. Applying these mortality rates to the total sheep and goat populations in the 65 infected countries, it is estimated that there are 37.4 million PPR-associated sheep and goat deaths each year (minimum 20.2 million, maximum 67.7 million), with a most likely total value of US$1,475 million, that could be as low as US$794 million or as high as US$2.7 billion (Jones *et al.,* 2016).

An understanding of the characteristics of the disease is critical for the implementation of control programs and the eradication of the disease. An important part of combating PPR is virus characterization, where the relationships between field isolates using reference and historical viruses are used to investigate the possible origins of the disease. The identification of the possible origin of the virus is one of the major factors contributing to control of the disease (Thomas *et al.*, 2006).

The molecular study of PPRV has been going on in Ethiopia and it has been understood that in addition to the endemic lineage, lineage III, linage IV has been found in different parts of the country (Muniraju *et al*., 2104; Alemu *et al*., 2019). The isolate KJ867541; Ethiopia/2010 is the first report of lineage IV in Ethiopia and is the first complete PPRV genome to be characterized from an outbreak in East Africa (Muniraju *et al*., 2014). However, sufficient genetic information of viruses from Ethiopia has not been available to determine the number of additional viral lineages in the country.

Few studies have been conducted in Ethiopia recently showing the burden of PPR in some parts of the country. PPR virus has been circulating in the highland areas of the country affecting the mixed crop-livestock farming system in the northern part of the country, Amhara Region (Alemu *et al.,* 2019). The virus was also identified from an outbreak occurred in the central part of the country, Bishoftu (Debre Zeit), where not only the virus was detected from the outbreak, but also a new lineage, lineage IV, was identified in the country for the first time (Muniraju *et al.*, 2014).

Despite the endemicity of the virus in the country, not enough study was conducted representing the pastoral systems and the lowland areas of the country. Therefore, these areas need more focus since significant proportion of the small ruminant population in the country is found in the pastoral farming system and 60% of the geographical area of the country being lowland.

Therefore, this study was designed with the following objective:

- ✓ To isolate and detect PPRV using molecular techniques from Outbreaks occurred in the pastoral areas of the country.
- ✓ To Check the test agreement of icELISA and RT-PCR

2. LITERATURE REVIEW

2.1. Definition

Peste des petits ruminants' virus (PPRV) was first identified in West Africa in the early 1940s and was initially thought to be a variant of Rinderpest virus (RPV) adapted to small ruminants. Subsequently, however, it was shown to be an antigenically and genetically distinct virus (Thomas *et al.*, 2006). PPR is an acute, highly contagious, notifiable and economically important Trans boundary viral disease of sheep and goat, which is listed by the World Organization for Animal Health (OIE). When the infection results in overt and acute disease, the most common outcome is death with case fatality rates that may exceed 90% in naïve populations (Albina *et al*., 2013).

2.2. Etiology

Peste des petits ruminant virus (PPRV) classified in the family Paramyxoviridae, genus *Morbillivirus* (OIE, 2013), Table 1.

Table 1: Classification of Morbilli virus

CLASSIFICATIONN	SPECIES
Order:*Mononegavirales*	
Family:*Paramyxoviridae*	
Genus: *Morbilli virus*	*Measles morbillivirus* *Canine morbillivirus* *Cetacean morbillivirus* *Feline morbillivirus* *Phocinemorbillivirus* *Rinderpestmorbillivirus* *Small ruminant Morbili v.*

Source: OIE, 2013.

The members of the genus *Morbillivirus*, classified within the order Mononegavirales, family *Paramyxoviridae*, sub-family *Paramyxovirinae* (Figure 1), are responsible for some of the most devastating diseases of humans and animals. Measles virus (MV) is

considered the typical virus for this genus, the name of which is derived from *morbilli*, the diminutive form of the Latin word for plague (*morbus*). This name was originally used to distinguish measles from smallpox and scarlet fever, which in former times were considered more serious diseases. In addition to MV, other Morbilliviruses that infect terrestrial mammals include Rinderpest virus (RPV), which causes cattle plague; Peste des petits ruminants virus (PPRV), which causes of sheep and goat plague, and Canine distemper virus (CDV), which infects many carnivore species including domestic dogs, mink and ferrets and can have serious consequences when endangered wildlife species are threatened (Thomas *et al.*, 2006).

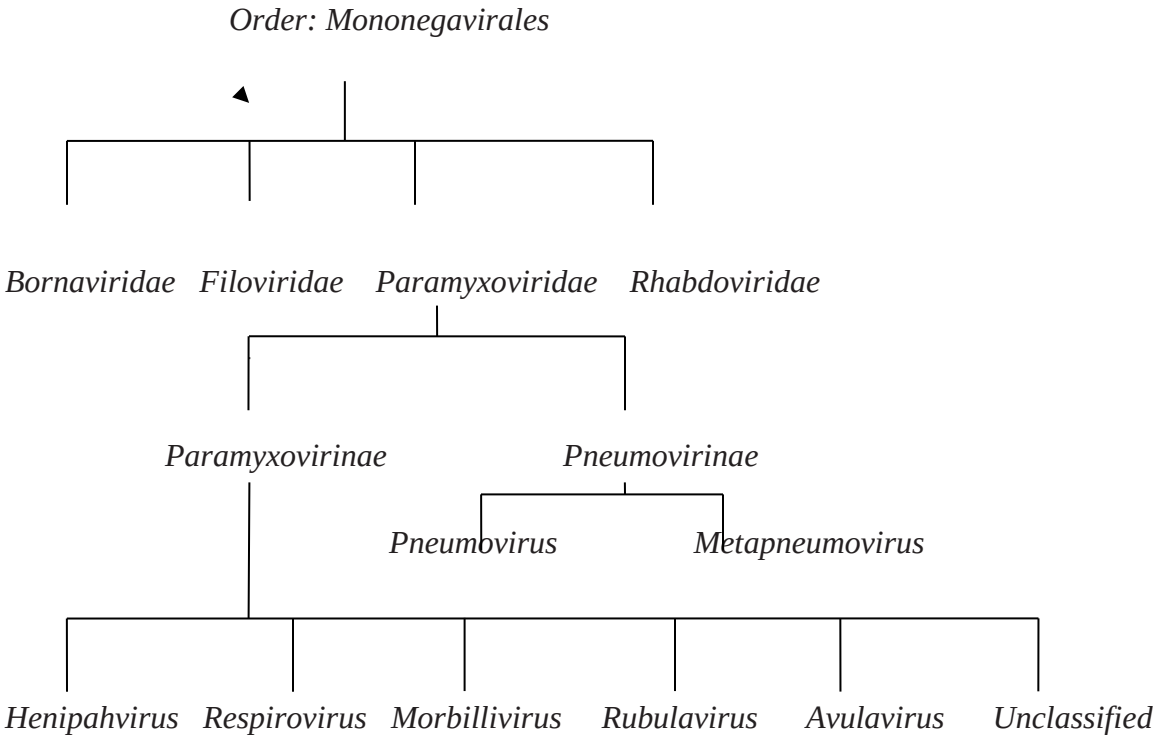


Figure 1: Classification of the Order Mononegavirales (Thomas *et al.*, 2006).

2.2.1. Morphology and biophysical characteristics of Peste des petits ruminant virus

Morbilli-viruses are linear, non-segmented, single stranded, negative sense RNA viruses with genomes 15-16 kb in length and 200nm in diameter. When viewed through electron microscope, morbilliviruses display the typical structure of *Paramyxoviridae*: a pleomorphic particle with a lipid envelope which encloses a helical nucleocapsid (Viral zone.com). The virus will be destroyed, if it is exposed to temperature at 50°C for 60

minutes and it is stable between pH of 5.8 and 10.0; thus virus inactivation is possible at pH<4.0 or >11.0. It can be killed by chemicals like alcohol, ether and common detergents and it is susceptible to most disinfectants, e.g. phenol, 2% sodium hydroxide. The virus survives for long periods in chilled and frozen tissues (OIE, 2013).

2.2.2. Genome structure and protein synthesis

All Paramyxovirus genomes contain sequences at their terminal extremities that act as promoters for transcription and replication. At the 3 ends of the genome RNA there is a leader region of 52 nucleotides that precedes the N-gene start and a similar length untranslated region (UTR) before the N-gene open reading frame (ORF) that contain promoter functions (Thomas *et al.*,2006). Virus members of this group have six structural proteins; the phosphoprotein (P), which associates with the polymerase (L for large protein) protein, the matrix (M) protein, the fusion (F) protein and the haemagglutinin (H) protein and two none structural proteins; C and V (Edward *et al.*, 2008).

Nucleocapsid (N-protein)

This is the major viral protein and possibly plays an important role in inducing antiviral immunity. Currently, the great interest in this protein is the use of its cDNA as a potential specific diagnostic probe (Edward *et al.*, 2008).

Haemagglutinin (H-protein)

The H-protein is responsible for attachment of the virus to the host cell. The biological activity of the H-protein is one of the criteria for classification of Paramyxoviridae. H-proteins are highly variable. Indeed, along with the P-protein, the H-protein is the least conserved of the morbillivirus proteins (Couacy-Hymann *et al*., 2007).

Fusion (F-protein)

The F protein is also highly conserved. Paramyxoviruses generate an inactive precursor (F0), which is cleaved by host cell enzymes to yield an active di-sulfide linked protein, F1–F2, and the cleavage site is also conserved. The F-protein is one of two glycosylated envelope proteins that constitute the peplomers or surface projections. Synthesized as a precursor, F0 is subsequently cleaved by cellular proteases into two disulfide-linked

polypeptides, F1 and F2. Proteolytic cleavage is believed to be essential for F-protein biologic activity.

The L-proteins

The L-proteins are multi-functional and, in addition to their polymerase activity, have methylation, capping and polyadenylation activities. Morbillivirus L-proteins have three highly conserved domains (designated A, B and C), separated by two hinge regions, which vary greatly between morbilliviruses (Chauhan *et al*., 2009).

C and V proteins

The C and V proteins of paramyxoviruses, although essentially non-structural, have been shown to have critical roles in infection. In RPV they were shown to be important for replication (Edward *et al.*, 2008). C-minus mutants showed growth defects in vitro, this being related to a reduced level of mRNA transcription. In contrast V-minus mutants were not defective in-vitro, but had an altered cytopathic effect and increased genome/antigenome RNA production. The C and V proteins of paramyxoviruses also act as interferon antagonists, modifying the cellular immune response to infection (Couacy-Hymann *et al*., 2007).

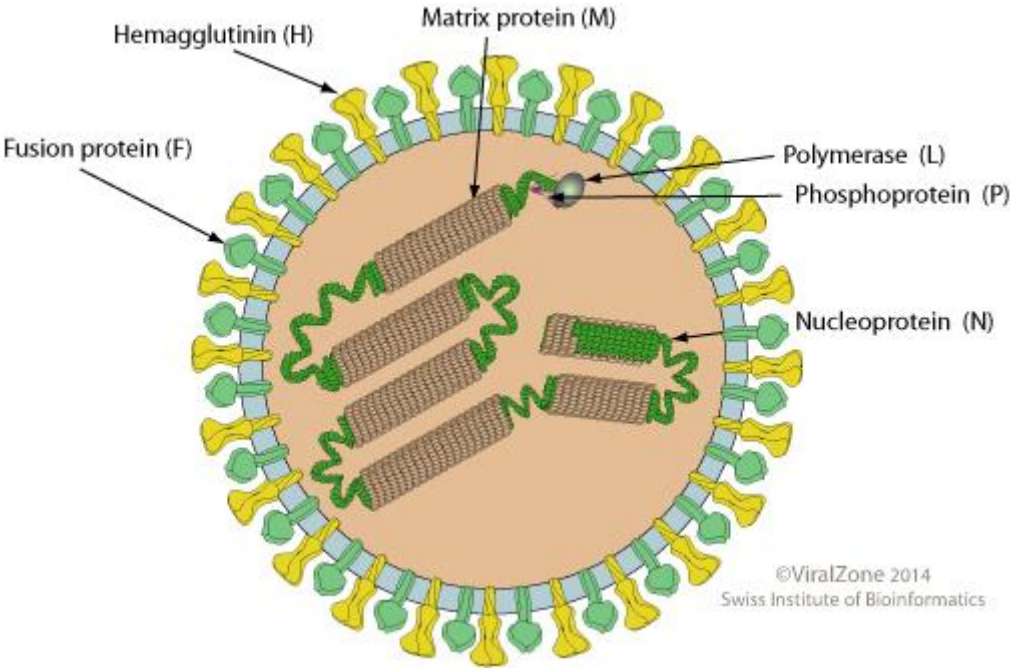


Figure 2: Morbilli virus structure (Viral zone.com)

2.2.3. Viral Replication

By definition, RNA viruses use RNA as genetic material and, thus, must use some relatively subtle strategies to replicate in a cell since the cell uses DNA. Ultimately, to express its genetic information, any virus must be able to present genetic information to the cell as translatable mRNA, but the way this happens with RNA viruses will depend on the type of virus and the nature of the encapsidated RNA (Edward *et al.*, 2008).

Viral infection begins with the liberation of the nucleocapsid into the cytoplasm of the cell due to the fusion of cellular and viral membranes. Then, viral RNA polymerase starts to transcribe the genome into messenger RNA (mRNA) in the order of arrangement of the genes, an order which is known for measles virus as follows: N - P/ C - M - F - H - L (from the 3' extremity of the genomic RNA to its 5' extremity). Results of gel-electrophoresis of the mRNAs induced by PPR virus are identical with those obtained for other Morbilliviruses (Brian and Hillar, 1996). They are translated in to different proteins by enzymes of the target cell. Each messenger gives rise to one protein; the second one, however, is translated in to two reading frames resulting in structural P-protein and non-structural C-protein. Once the concentration of the viral proteins reaches a sufficient amount, polymerase turns to the synthesis of new genomes, each of which is surrounded by N, P and L proteins to form nucleocapsids. These migrate to a region of the membrane, where M, F and H proteins are already present. At this site a bud forms and expands until it detaches from the target cell as a mature virus. The budding process starts 12 hours after infection of sheep cells by PPR V and continues until the seventh day.

Budding of morbilliviruses may occur at specialized membrane regions for which the M-protein has more affinity; for example lipid raft microdomains are the potential locations for MV assembly and it is possible that the apical domains are richer in these membrane structures. Actin filaments, responsible for cellular transport, also are required for virus budding and it has long been known that destruction of actin filaments prevents MV budding (Thomas *et al.*, 2006).

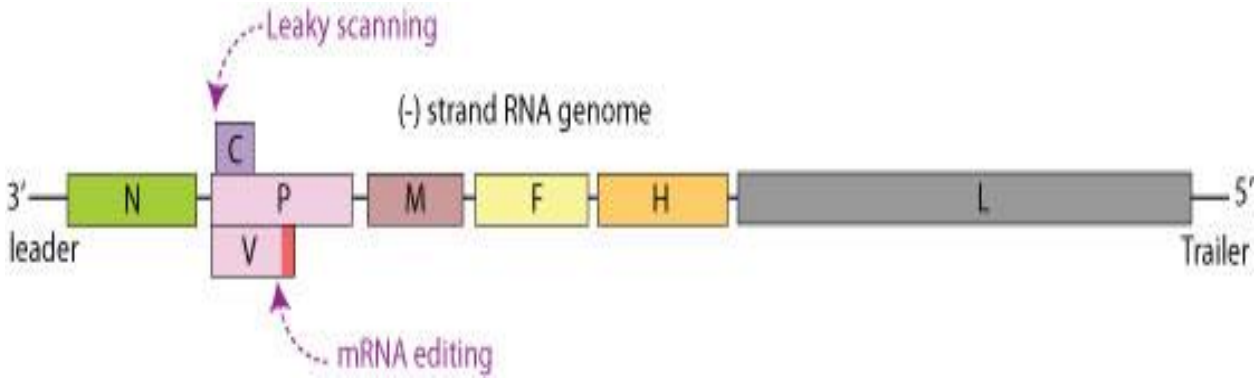


Figure 3: Morbilli virus genome structure and arrangement

2.3. The linage distribution and dynamics

PPR occurs in Africa except in Southern Africa, in the Arabian Peninsula, throughout most of the Near East and Middle East, and in Central and South-East Asia (OIE, 2013, Zixiang *et al.*, 2016). Lineage III is endemic in Ethiopia and has been found in east Africa. However, the lineage distribution is currently changing (Fakri *et al*., 2016). The isolate KJ867541; Ethiopia/2010 is the first report of lineage IV in Ethiopia and is the first complete PPRV genome to be characterized from an outbreak in East Africa. This isolate was derived from the intestine of a goat suffering from severe clinical disease during the 2010 outbreak following the procurement of 50 male goats aged 6–8 months from Debre Zeit market showing no signs of ill health (Muniraju *et al*., 2014). Currently, the National Veterinary Institute (NVI) produces live attenuated PPR vaccine using PPR75/1 (LK6 Vero74) strain (Kula, 2016).

2.3.1. The antigenic variation

Analysis of a small sequence of the PPRV nucleoprotein (NP) or F gene permits classification of the strains of the unique serotype of circulating PPRV into 4 genetically distinct lineages (Gian *et al*., 2013).

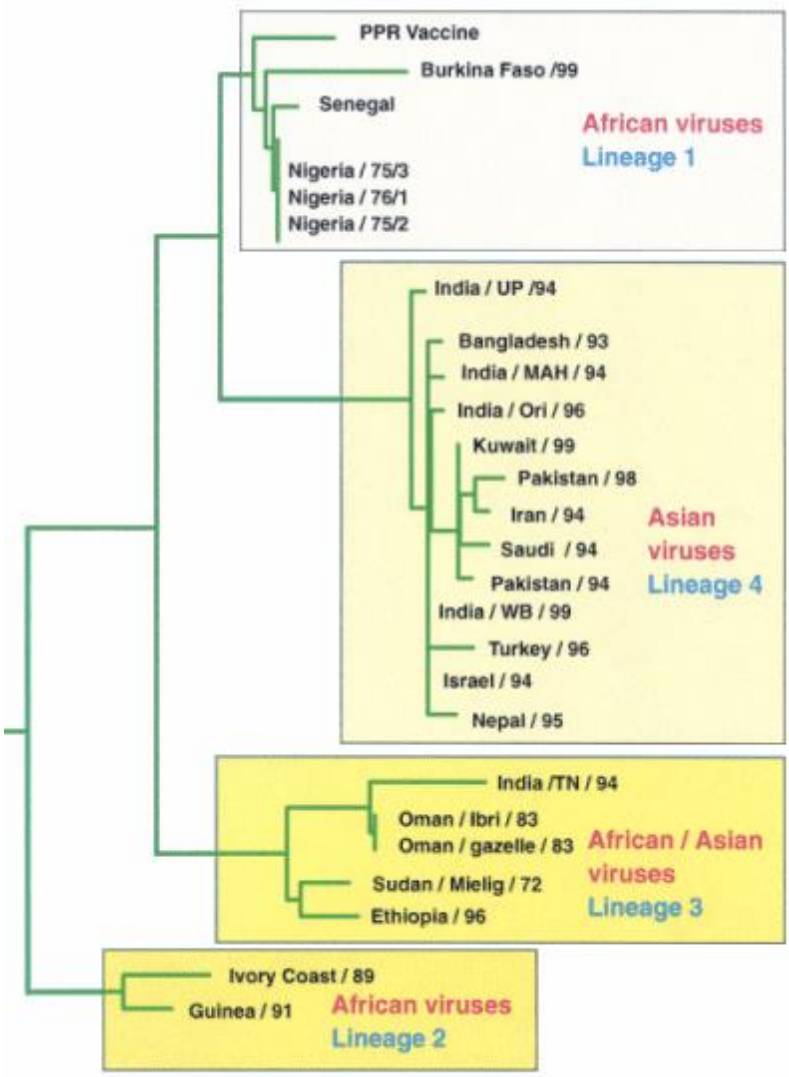


Figure 4: Phylogenetic relationships of the Peste des petits ruminants' virus isolates (Albina *et al*., 2013).

2.4. Susceptible hosts

Peste des petits ruminants affects mainly sheep and goats and occasionally wild small ruminants. Based on the fact that PPR has been reported on a few occasions in camels, cattle and buffaloes, those animal species are considered to be susceptible although their potential role in the circulation of PPR virus (PPRV) has not been formally established (OIE, 2013).

The levels of virulence for PPR varied between sheep and goats. There is variation in the inherent resistance of different breeds of sheep and goats to PPRV. Goats are highly susceptible while milder forms of the disease may occur in sheep and partially immune goat populations (Couacy-Hymann *et al*., 2007).

2.5. Pathogenesis

The pathogenesis of PPRV is poorly understood, most of the knowledge is based on comparison with related Morbilliviruses. The pathogenesis in sheep and goats, in general, is similar to those of Rinderpest except that the disease is more acute in onset, especially in goats and follows a more rapid course. Another difference is the marked involvement of the respiratory tract; affected animals have dyspnea, hyperpnea and coughing. There is also a marked serous to mucopurulent nasal and ocular discharge and erosion or ulceration of the pharyngeal epithelium (Thomas *et al.*, 2006).

Many animals with PPR show abnormally rapid or labored breathing and a productive cough. By this stage, the animal is apathetic, with labored breathing and an unwillingness to move. Convalescence, if it occurs, takes several weeks (Abraham, 2005). Any animal that is pregnant at the time of infection will abort. The white blood cell count slowly returns to normal and the oral lesions heal over a period of two to three weeks. This transient loss of white cells and the generalized immunosuppression that can go on for even longer means that the animal is susceptible to activation of latent or chronic infections (e.g. with parasites) or to secondary infection by other pathogens. The virus infection, on the other hand, completely resolves in recovered animals, and there is no persistent infection or carrier state (Thomas *et al.*,2006; Kula, 2016).

PPR virus, like other morbilliviruses, was lymphotropic and epitheliotropic. Consequently, it induces the most severe lesions in organ systems rich in lymphoid and epithelial tissues and infection usually results in conjunctivitis, rhinotracheitis, ulcerative stomatitis, gastroenteritis and pneumonia. Clinical signs usually develop within 3–5 days following establishment of pyrexia. As disease progresses, mucosal hyperaemia, mucoid nasal discharges, anorexia and diarrhea are observed (Albina *et al*., 2013). The outcome of

the infection depends on the ability of the animals to mount specific immune response to PPRV. Factors such as co-infection with pre-existing parasitic organisms and nutritional status of the animal may also contribute in determining the disease severity and hence morbidity and mortality rates. Death may occur from severe diarrhea and dehydration, before respiratory lesions become severe, or is hastened by concurrent diseases such as pneumonic pasteurollosis, coccidiosis or coliform enteritis (Couacy-Hymann *et al.*, 2007).

2.6. Diagnosis

2.6.1. Clinical signs of the disease

The clinical disease resembles Rinderpest in cattle. It is usually acute and characterized by pyrexia, serous ocular and nasal discharges, diarrhea, pneumonia, and erosive lesions on different mucous membranes particularly in the mouth. At necropsy, erosions may be noted in the gastrointestinal and urogenital tracts. The lungs may show interstitial bronchopneumonia and often secondary bacterial pneumonia. PPR can also occur in subclinical form (Thomas *et al.*, 2006).

The incubation period is typically 4–6 days but may range between 3 and 10 days. The clinical disease is acute, with pyrexia up to 41°C that can last for 3–5 days; the animals become depressed, anorexic and develop a dry muzzle. Serous oculo-nasal discharges become progressively mucopurulent and, if death does not ensue, persist for around 14 days. Within 4 days of the onset of fever, the gums become hyperaemic, and erosive lesions develop in the oral cavity with excessive salivation. These lesions may become necrotic. Watery blood-stained diarrhoea is common in the later stage. Pneumonia, coughing, pleural rales and abdominal breathing also occur. The morbidity rate can be up to 100% with very high case fatality in severe cases. However, morbidity and mortality may be much lower in milder outbreaks, and the disease may be overlooked. A tentative diagnosis of PPR can be made on clinical signs, but this diagnosis is considered provisional until laboratory confirmation is made for differential diagnosis with other diseases with similar signs (OIE, 2013).

PPR covers three essential forms: Per acute form, an acute form and a mild form. Acute PPR first results in a sudden dullness of infected animals, with high fever and inappetence. One or two days later, congestion of oral, ocular and nasal mucosae leads to serous discharges that later on become more abundant and mucopurulent. Bronchopneumonia, revealed by productive cough and dyspnea, and diarrhea usually appears 3 days after the oral lesions. As a consequence of pneumonia and dehydration caused by diarrhea, severely affected animals may die within 5–10 days after the onset of clinical signs. Abortions are often observed during PPR outbreaks (Abraham, 2005). The *peracute* form of the disease starts often after a short incubation period of 2 days with a sudden high rise in body temperature up to 40–42°C accompanied by serous oculo-nasal discharges, depression, dyspnoea, anorexia and constipation become congested and occasionally eroded. Affected animals develop profuse watery diarrhoea and die within 4–6 days after the onset of fever. In s*ubacute* forms less severe illness is observed after an incubation period of 6 days and low-grade fever. Affected animals do not display all the clinical signs described above and the mortality rate is much lower. Many animals will recover after an illness of 10–14 days. *Subclinical* infections are characterized by seroconversion alone (Thomas *et al.*, 2006).

PPR must be confirmed by laboratory methods, as rinderpest, bluetongue, foot and mouth disease and other erosive or vesicular conditions as well as contagious caprine pleuropneumonia, can cause clinically similar disease.

2.6.2. Laboratory Diagnosis

Beside clinical diagnosis based on lesion identification, in the early stage of infection, PPR virus or viral antigen can be detected using several diagnostic techniques.

A) Techniques for identification of the agent

I) Agar gel immunodiffusion (AGID)

AGID is a very simple and inexpensive test that can be performed in any laboratory and even in the field. Standard PPR viral antigen is prepared from infected mesenteric or

bronchial lymph nodes, spleen or lung material and ground up as 1/3 suspensions in buffered saline. These are centrifuged at 500*g* for 10–20 minutes, and the supernatant fluids are stored in aliquots at –20°C. The cotton material from the cotton bud used to collect eye or nasal swabs is removed using a scalpel and inserted into a 1 ml syringe. With 0.2 ml of phosphate buffered saline (PBS), the sample is extracted by repeatedly expelling and filling the 0.2 ml of PBS into an Eppendorf tube using the syringe plunger. The resulting eye/nasal swab extracted sample, like the tissue ground material prepared may be stored at –20°C until used and may be retained for 1–3 years. Negative control antigen is prepared similarly from normal tissues. Standard antiserum is made by hyper-immunizing sheep with 1 ml of PPRV with a titer of 104 TCID50 (50% tissue culture infective dose) per ml given at weekly intervals for 4 weeks. The animals are bled 5–7 days after the last injection (OIE, 2013).

II) Counter immuno-electrophoresis (CIEP)

Counter immuno-electrophoresis is the most rapid test for viral antigen detection. It is carried out on a horizontal surface using a suitable electrophoresis bath, which consists of two compartments connected through a bridge. The apparatus is connected to a high-voltage source. Agar or agarose (1–2%) dissolved in 0.025 M barbitone acetate buffer is dispensed on to microscope slides in 3-ml volumes. From six to nine pairs of wells are punched in the solidified agar. The reagents are the same as those used for the AGID test. The electrophoresis bath is filled with 0.1 M barbitone acetate buffer. The pairs of wells in the agar are filled with the reactants: sera in the anodal wells and antigen in the cathodal wells. The slide is placed on the connecting bridge and the ends are connected to the buffer in the troughs by wetted porous paper. The apparatus is covered, and a current of 10–12 milliamps per slide is applied for 30–60 minutes. The current is switched off and the slides are viewed by intense light: the presence of 1–3 precipitation lines between pairs of wells is a positive reaction. There should be no reactions between wells containing the negative controls (Gian *et al*., 2013).

III) Immunocapture enzyme-linked immunosorbent assay (icELISA)

The icELISA using two monoclonal antibodies (MAb) rose to the N-protein allows a rapid identification of PPRV. The instructions provided by kit supplier should be followed (OIE, 2013).

V) Lateral flow device (LFD) test

It is a rapid screening test/pen-side test. The LFD-based PPRV test is based on the specificity and affinity of monoclonal antibody. This antibody recognizes the PPRV H-protein. The instructions provided by kit supplier should be followed (Purbright Ltd.).

B) Nucleic acid recognition methods

I) Polymerase Chain Reaction (PCR) techniques

Reverse transcription PCR (RT-PCR) techniques based on the amplification of parts of the N and F protein genes have been developed for the specific diagnosis of PPR. This technique is 1000 times more sensitive than classical virus titration on Vero cells with the advantage that results are obtained in 5 hours, including the RNA extraction, instead of 10–12 days for virus isolation. The two most commonly used protocols are given in some detail below. A multiplex RT-PCR, based on the amplification of fragments of N and M protein genes, has been reported. Another format of the N gene-based RT-PCR has also been described. Instead of analyzing the amplified product, the amplicon, by agarose gel electrophoresis, it is detected on a plate by ELISA through the use of a labeled probe. This RT-PCR-ELISA is ten times more sensitive than the classical RT-PCR. In recent years, nucleic acid amplification methods for PPR diagnosis have been significantly improved with quantitative real-time RT-PCR. This method is also ten times more sensitive than the conventional RT-PCR, as well as minimizing the risk of contamination. The application of nucleic acid isothermal amplification to PPR diagnosis has also been described. The sensitivity of this assay seems to be similar to that of the real-time RT-PCR. This assay is simple to implement, rapid and the result can be read by naked eye (OIE, 2013).

1/ RT-PCR for the diagnosis of PPRV based on the amplification of part of the N-gene

The RT-PCR gives an amplification product of 351 bp. 10µl of these products are analyzed by electrophoresis on 1.5 % agarose-gel. For all positive results, 40µl of the final product may be directly used for sequencing.

2/ RT-PCR for the diagnosis of PPRV based on the amplification of part of the F-gene

Analyze 10µl of the reaction product by agarose gel electrophoresis using a 2% agarose gel in either TBE or TAE buffers. If present, PPRV RNA will be amplified to give a DNA fragment of 371 bp.

C) Culture and isolation methods

Even when diagnosis has been carried out by rapid techniques, the virus should always be isolated from field samples in tissue cultures for further studies. Viruses are obligate intracellular parasites that require living cells in order to replicate. Cultured cells, eggs and laboratory animals could be used for virus replication in order to get isolate of that virus.

PPRV may be isolated in primary lamb kidney/lung cells and some cell lines (Vero, B95a). Unfortunately, PPRV isolation using such cells is not always successful on first passage and may require multiple blind passages. Recently derivatives of cell lines (Vero, CV1) expressing the morbillivirus receptor, the signaling lymphocyte activation molecule (SLAM or CD150), have been developed that can enable isolation of field viruses from pathological specimens in less than 1 week, without requirement for blind passages. These include a derivative of the monkey cell line, CV1, expressing goat SLAM and derivatives of Vero cells expressing dog SLAM. Monolayer cultures are inoculated with suspect material (swab material, buffy coat or 10% tissue suspensions) and examined daily for evidence of cytopathic effect (CPE). The CPE produced by PPRV can develop within 5 days and consists of cell rounding and aggregation culminating in syncytia formation in lamb kidney cells and cell lines expressing SLAM. In unmodified Vero cells, it is sometimes difficult to see the syncytia. If they exist, they are very small. However, small syncytia are always seen in infected Vero cells stained with

haematoxylin and eosin. Syncytia are recognized by a circular arrangement of nuclei giving a „clock face" appearance. Cover-slip cultures may show CPE earlier than day 5. Some cells may contain intracytoplasmic and intranuclear inclusions, others may be vacuolated. Similar cellular changes may be seen in stained histopathological sections of infected tissues. After 5–6 days, blind passages should always be carried out as CPE may take time to appear (OIE, 2013).

D) Serological tests

The demonstration of antibodies in PPRV infected goats and sheep can be used to support a diagnosis based on clinical signs, but such antibodies may also arise from vaccination with any of the current PPRV vaccines. Tests that are routinely used are the virus neutralization (VN) test and the competitive ELISA.

I) Virus neutralization

Recommended for international trade, this test is sensitive and specific, but it is time-consuming. The standard neutralization test is now usually carried out in 96-well microtitre plates although roller-tube cultures may also be used. Vero cells are preferred, but primary lamb kidney cells may also be used (OIE, 2013).

II) Competitive enzyme –linked immunosorbnt assay

Several competitive ELISAs (C-ELISA) have been described, based on the use of MAbs that recognize virus proteins. They are of two types: those where the Mab recognizes the N-protein and use recombinant N-protein produced in baculo-virus as the antigen and those with a viral attachment protein (H) specific MAb and antigen consisting of purified or part-purified PPRV (vaccine strain). All the assays work on the principle that antibodies to PPRV in test sera can block the binding of the MAb to the antigen (OIE, 2013).

2.7. Control and prevention

Sheep and goats which have recovered from a PPR infection appear to be protected against a subsequent infection for the rest of their lives. Neutralizing antibodies for PPRV

were found in sheep and goats up to three years after vaccination with the attenuated PPRV vaccine strain Nigeria 75/1(Baazizi *et al*., 2017). Other attenuated PPR vaccines that have been developed in India have been shown to produce strong immunity too. All this information indicate that PPR is a disease that can be well controlled, even eradicated following mass and well-planned vaccination campaign as that was done for rinderpest (OIE,2013).

Effective live attenuated PPR virus vaccines are now widely available. Since the global eradication of rinderpest, heterologous vaccines should not be used to protect against PPR (OIE, 2013).

2.8. Status in Ethiopia

PPR was clinically suspected for the first time in Ethiopia in 1977 in a goat herd. Serological evidence was reported in 1984 and later confirmed in 1991 with Complementary DNA **(**cDNA) probe in lymph nodes and spleen specimens collected from an outbreak in a holding area near Addis Ababa. PPR is among the commonest of the diseases that affect small ruminants entailing a huge economic loss as it is listed as trans- boundary diseases affecting the economy of the countries through limiting international trade of animals and animal products (Subir and Hemayeatul, 2011) . A global and national control and eradication strategy has been prepared and is being implemented in pastoral lowland and highland-lowland interface areas in the country in collaboration with FAO (FAO, 2013).

PPR is considered as one of the main constraints in augmenting the productivity of small ruminants in developing countries and particularly severely affects poor farmer's economy (Balamurugan *et al*., 2014). PPRV has caused numerous serious epidemics in small ruminant populations across sub-Saharan Africa, the Middle East and major parts of the Indian subcontinent. (Muniraju *et al.*, 2010, Nanda *et al*., 2002). PPR was introduced to Ethiopia in 1989 in the Southern Omo River Valley from where it moved east to Borana then northwards along the Rift Valley to Awash. The disease then spread

northwards into the central Afar Region and eastwards into the Ogaden (Abraham *et al*., 2005). After the first confirmed cases of PPR in Ethiopia, the disease is continuously affecting small ruminant production and thus contributing to food insecurity, particularly, in vulnerable regions of the country (Waret-Szkuta *et al*., 2008).

Lineage III is endemic in Ethiopia and has been found in the whole of East Africa. However, the lineage distribution is currently changing. The isolate KJ867541; Ethiopia/2010 is the first report of lineage IV in Ethiopia and is the first complete PPRV genome characterized from an outbreak in East Africa. This isolate was derived from the intestine of a goat suffering from severe clinical disease during the 2010 outbreak following the procurement of 50 male goats aged 6–8 months from Debre Zeit market showing no signs of ill health (Muniraju *et al*., 2014).

3. MATERIAL AND METHODS

3.1. Study area

The geographic focus of the present study was the lowland area of Ethiopia, mainly the pastoral areas such as Somali Region and Afar Region, and some lowland-highland interface areas of western Amhara and eastern Tigray bordering Afar Region and parts of Benishangulgumuz from the western part of the country, which is bordering Sudan and South Omo Zone of SNNPR. A total of nine outbreaks were considered in the present study which occurred in the eight districts (woredas) of the country which included namely: Daweharawa and Habru (Amhara Region), Telalak and Chifra (Afar Region), Belojigamfure (Benishangulgumuz Region), Enderta (Tigray Region), Konso (SNNP Region), Hadigala and Jigjiga (Somali Region).

Daweharawa District

Daweharawa District is found in Amhara Region, Oromia Special Zone, bordering Artumafuri District in south, Dawachafa District in west, Dalifage District of Afar Region in the east and Argoba Special District of Afar in northern part. The small ruminant population of the district is estimated to be 70,500 with sheep and goat counting 20,500 and 50,000, respectively. The district consists of all agro-ecologies dominated by lowland. The lowland covers around 89% (*Kola*), mid-land 10% (*Woyna-dega*) and highland 1% (*Dega*). The district is located on altitude ranging from 1000 meters – 2500 meters above sea level with mean temperature ranging from 24^{oC}-C-28^{o}C. The residents of the district are mainly pastoralists that relay on animals for their day to day livelihood (WoA, 2017).

HabruDistrict

Habru District is one of the districts in Amhara Region. It is found in North Wollo Zone bordered by Mille River in south, Gubalafto District in west, Kobo District in the north and Afar Region in the east. The average elevation of the district is between 700- 1900

meters above sea level. The district is one of the draught prone districts of the country. The total population of the district is estimated to be 192,742 (CSA, 2014)

Hadigala District

Hadigala District is found in Somali Region, Sitti Zone bordering Djibouti in the north, Aysha District in the east, Geblelu District in the west and with Shinile and Dambel Districts in south. The residents are 100% pastorals (CSA, 2014)

JigjigaDistrict

Jigjiga District is found in Somali Region, Jigjiga Zone bordering Kabribeyah District in south, Awbare District in east, Gursum and Babile Districts of Oromia Region in the northern. The small ruminant population found in the district is 996, 708 sheep and 916, 193 goat, respectively (CSA, 2014)

Telalak District

Telalak District is found in Afar Region, Zone Five, bordering Kemise District of Amhara Region in the west, Ada'ar District in the east, Dawe District in south and Gewane District in the north. The small ruminant population of the district is estimated to be 257,396 with much part taken by goats. The Agro-ecology of the district is more of lowland with a mean altitude of 800 meters above sea level. The residents found in the district are 50% agro-pastoralists, who cultivate by irrigation using rivers found in the district and the rest 50% being solely pastorals (WoPADO, 2017).

Chifra District

Chifra District is found in Afar Region, Zone one. The District is bordered by Mille District in the south and Amhara Region in south and Zone four of Afar Region on the East .The average elevation of the district is 825 meters above sea level. The total population of the district is estimated to be 91,000. The district is fully pastoral, majority of the population living in rural areas of the district (CSA, 2014).

Belojigamfure District

Belojigafure District is found In Benishangulgumuz Region , Kemashi Zone, bordering Yaso District of B/Gumuz, Halolumu District and Gidaayana Districts of Oromia Region in the northern and with Sasiga District of Oromia and Kemashidistrict of B/Gumuz Region in the western side. The southern part shares border with Diga and Gimbi Districts of Oromia Region. Geographically, it is located between N 09^{o} 21' 37.5" latitude and E 036^{o} 11' 20.4" longitude with the annual rainfall of 700-750ml. The District mainly has semi-arid ecology and is found 1367 meters above sea level with 31^{o}C of mean temperature. The total population of small ruminants in the district is 30,881. In addition, the District consists two major rivers namely Dedesa and Anger (WoA, 2017).

Konso special District

Konso Special District is one of the districts in SNNPR. The District has latitude of 5^{0}15’ N and longitude of 37029’E. The District is located 500-2500 meters above sea level. The total population of the District is estimated to be 186,000 (CSA, 2014).

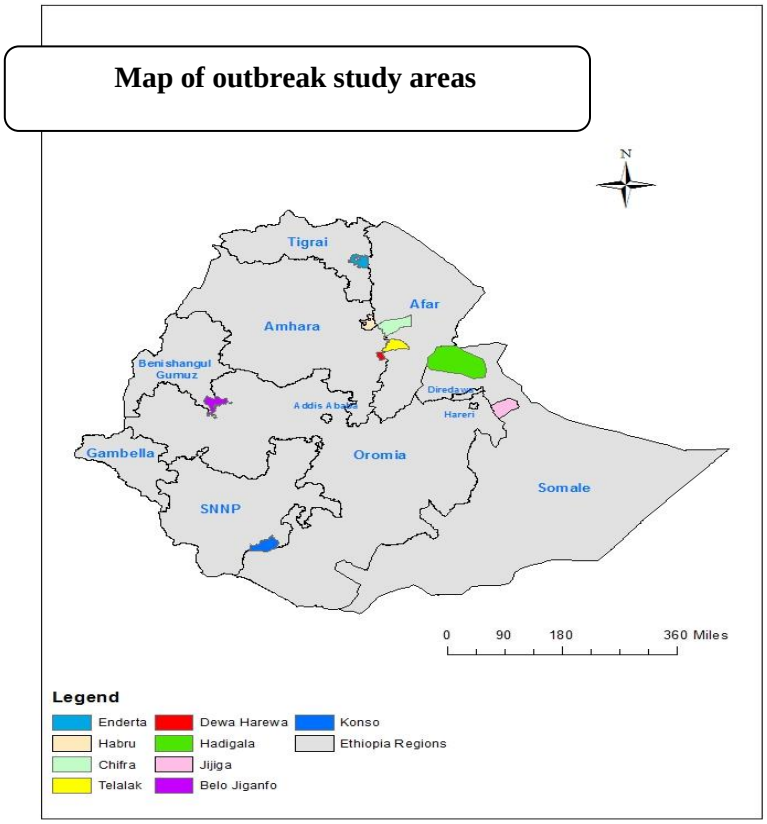


Figure 5: Map showing districts where PPR outbreaks were investigated during the study

3.2. Study population

The study population was sheep and goat flocks, where recent PPR outbreaks has been reported and confirmed positive by pen-side test kit. Moreover, the additional inclusion criterion was the population has no history of receiving vaccination for PPR as confirmed by district veterinarians. The sampling was done between the 1st and the 4th week of the outbreak occurrence. Animals at all age which were displaying the clinical signs of the diseases were sampled randomly from the pen-side positive flock.

3.3. Study design and sampling

The study design used was cross-sectional investigation on PPR outbreak reports through collecting epidemiological data and laboratory specimens. The sampling method used was purposive sampling employed between December 2017 and May 2019. A proper information collection channel was established at the beginning of the study and used to collect information about the occurrences of the disease outbreaks from different lowland areas of the country. Six regional veterinary laboratories (RVL) (Semera RVL, Jigjiga RVL, Kombolcha RVL, Asosa RVL, Mekele RVL and Soddo RVL) and regional animal health offices of the respective regions mentioned above were involved in the process of investigation by informing about the suspected PPR outbreak and report any type of outbreaks in the sheep and goat population to the National PPR Control and Eradication Coordination Office of the Ministry of Agriculture, which is based in Addis Ababa, supported by the Europoian Union and technically supported by FAO-Ethiopia. The National Coordination Office established BCOs (Branch Coordination Offices) in the regions where the project is operating. These BCOs have been used as focal points to collect information from the Districts targeted by this study. When an active outbreak was reported by regional animal health officers or if the BCOs reported a pen-side positive flock, a field investigation was conducted on the location immediately. Clinical and epidemiological information was recorded using investigative interview with the owners and sick animals were clinically examined and specimens were collected from the animals which are displaying the clinical signs actively (Table 2).

Table 2: Result of flocks for PPR pen-side test

Region	District	Flock size	No. of sick	No. of dead	investigation of the outbreak	Pen-side result
B/Gumuz	Belojigamfure	200	80	12	2weeks	Positive flock
Amhara	Daweharwa	15	12	1	1week	Positive flock
	Habru	47	1	0	1week	Positive flock
Afar	Telalak	500	2	0	3weeks	Not tested
	Chifra	250	2	0	1week	Positive flock
Somali	Hadigala	320	25	2	4weeks	Positive flock
	Jigjiga	50	7	0	2weeks	Positive flock
SNNPR	Konso	300	25	1	2weeks	Positive flock
Tigray	Enderta	20	3	0	1week	Positive flock

Based on this, a total of nine outbreaks were investigated from six regions: 2 from Amhara, 1 from B/Gumuz, 2 from Afar, 1 from Tigray 1, 1 from SNNPR and 2 from Somali. Out of the nine investigated outbreaks, eight were PPR positive for pen-side test. A total of 60 nasal and eye swabs were collected from 60 animals, 12 sheep and 48 goats, respectively (Table 3).

Table 3: Study area and number of samples collected

Region	District	Kebele	No. of sample
B/Gumuz	Belojigafure	01	10
Afar	Telalak	Kone	2
	Chifra	01	2
Amhara	Daweharawa	Uraneslama	8
	Habru	Meerkota	1
Somali	Hadigala	Gebi	18
	Jigjiga	05	6
SNNPR	Konso	Albisame	10
Tigray	Enderta		3
		Total	60

3.4. Sample collection and laboratory diagnosis

Suspected PPR outbreaks were tested at a flock level. A flock is a group of small ruminants owned by one person with the size ranging from 15 to 500 heads and suspected active cases of PPR were tested using PPR pen-side test kit (Peste-test rapid test for PPRV infection) by Brach Coordination Officers of the respective areas. The pen-side test was not done for individual animals, rather it was done for representative sick animals and if the result of one or more samples were found positive, the whole flock is considered positive for PPR. In about 1-4 weeks, after the outbreak has been reported and/or confirmed by pen-side test, samples were collected and examined with different molecular techniques for virus/antigen detection and viral nucleic acid detection.

A total of 60 swab samples were collected from sick sheep and goats and samples were taken from flocks, in which at least one animal reacted positive for the pen-side test. Swab samples were collected from sick animals' body fluids such as nasal discharge, fluid discharges of the eyes and from lesions on the gum. The samples were labeled accordingly to allow identification of each animal and flock and kept in a viral transport medium (VTM) prepared by NAHDIC and kept in an icebox before being transported and stored to the diagnostic laboratory. Finally, the swab samples were shipped in an ice box chilled on ice packs to the National Animal Health Diagnostic and Investigation Center (NAHDIC) at Sebeta, where virus isolation and molecular detection were carried out.

3.4.1. Cell culture

Vero cells were revived from liquid nitrogen and re-cultured in $25cm^2$ tissue culture flask. The confluent flask was then sub-cultured to multiple $25cm^2$ TC flasks and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37^oC in a humidified incubator at 5% CO_2 (Annex 1).

Nasal and ocular swabs were collected from active cases in sterile universal tubes containing virus transport medium (VTM). The swabs were macerated well in the transport medium itself used for collection. The resulting suspensions were transferred to a centrifuge tube and centrifuged at 3000-5000 rpm for 20 min. The supernatants were collected, filtered with 0.22um pore size syringe filter and inoculated in to the suitable cell lines (OIE manual, 2013) (Annex 2).

Samples were taken from 5 from BelojiGamfure outbreak, and 3 samples from Enderta outbreak for purpose of studying Cytophatic effect of the PPRV. Two passages were conducted, the first passages shows a little to no cytopathic effect and second passage was done after a week where a visible cytophatic effect of the virus was seen. The result was seen starting from the 2nd day of second passage and it was microscopically examined daily for evidence of cytopathic effect (CPE).

3.4.2. Immunocapture enzyme-linked immunosorbent assay (icELISA)

The icELISA using two monoclonal antibodies (MAb) rose to the N protein allows a rapid identification of PPRV. The instructions provided by kit supplier was followed (OIE, 2013). The sandwich ELISA kit (ID Vet) was used to detect positive samples. It uses an anti-nucleoprotein (N) capture antibody and an anti-N monoclonal antibody for revelation. The kit was made with the wells coated with an anti-PPRV-N antibody. Samples to be tested and controls were added to the micro wells. In each well was added 25µl dilution buffer and the same amount of negative and positive controls to well A1 and B1 for the negative and C1 and D1 for the positive control, and 25µl of sample to the remaining wells. The plate was agitated and incubated for 45 minutes at 37^{o}c. After the incubation, it was washed 6 times using wash solution and 100µl of conjugate was added to each well and incubated for 30 minutes at 21^{o}C. After the second incubation, the well were again washed 6 times using 300µl wash solution. 100µl of substrate was added to each well and incubated for 15 minutes at 21^{o}C in a dark place/room. Finally,100µl stop solution was added to each well and the result was read and recorded the O.D. at 450 nm. (ID. Vet,Innovative Diagnostics manual) (Annex 3).

3.4.3. Polymerase chain reaction (PCR)

A total of 60 swab samples collected from sheep (n=12) and goat (n-48), from all age groups comparing of 34 male and 26 females. The collected samples were examined for the presence of PPRV RNA by the one step Reveres Transcription Polymerase Chain Reaction (RT-PCR) assay, (Table 4).

Table 4: Samples examined using RT-PCR technique

Districts	**Kebele**	**Spp**		**Sex**		**Total sample Collected**
		Caprine	**Ovine**	**Male**	**Female**	
Belojigamfure	01	10	0	6	4	10
Daweharwa	U/ selama	7	0	4	3	7
Telalak	Gublo	2	0	2	0	2
Hadigala	Gebi	14	4	11	7	18
Jigjiga	05	3	4	5	2	7
Chifra	01	2	0	1	1	2
Enderta	Dergajen	3	0	1	2	3
Konso	Albisame	6	4	4	6	10
Habru	Merkota	1	0	0	1	1
	Total	**48**	**12**	**34**	**26**	**60**

RNA extraction from samples was done using a commercial RNA extraction kit (Qiagen® Qiamp viral RNA mini kit), as per the manufacturer's instructions (Annex 4). Reverse Transcription- Polymerase Chain Reaction (RT-PCR) was performed for the N-gene of PPRV using the QIAGEN® one step RT-PCR kit as per the manufacturer's instructions. The reverse transcription and PCR were carried out sequentially in the same tube. The RNA obtained was converted to cDNA using a reverse transcriptase enzyme. The cDNA was amplified using PPRV specific NP3 and NP4 primers as previously described by.

The master mix contained the following reagents: 7 .5µl of RNase-free water, 5 µl of 5X PCR buffer, 1 µl of dNTPS mix (10mM each), 1 .5µl of each primer; NP3: (5′-GTC TCG GAA ATC GCC TCA CAG ACT - 3′) and NP4: (5′ CCT CCT CCT GGT CCT CCA GAA TCT 3′) at final concentration of 6 µm, 5 µl of Q solution and 1 µl of Qiagen enzyme mix.

The amplification was carried out with the final reaction volume of 25 µl containing 22.5µl of the prepared master mix and 2.5µl of RNA template. This mixture was submitted to a thermal cycling profile of initial reverse transcription at 50 °C for 30 min, PCR activation at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 30s, annealing at 60 °C for 30s, extension at 72 °C for 1 min and final extension at 72oc for 5 min in an Applied Biosystem 2700/2720 Thermal cycler conventional PCR machine (Annex 5).

Each PCR product (amplicon) of 10 µl were analyzed by gel electrophoresis at 120v/80 mA for 60 min on 1.5% of agarose gel in Tris-Borate-ETDA (TBE) buffer. The gel was stained with ethidium bromide and the DNA bands were visualized by UV trans illuminator and the image was transferred to a computer , reading based on the fact that PPR virus positive sample made bands at 351 bp according to the ladder for the test which was set in 100 bp gap (Annex 6)

3.4.4. PCR product purification and sequencing

Purification of PCR product performed by DNA purification kit (Wizard® SV Gel and PCR Clean-Up System) per manufacturer instruction. The purified product sent to LGC genomics for sequencing. The sequenced data obtained from the LGC genomics was fed to chromate viewer applied bio system software.

3.5. Data management and analysis

3.5.1. Test results and other information analysis

Descriptive statistics was used to summarize the data. The data gathered from the outbreak occurrence site and the laboratory tests result were filled in Microsoft Excel 2013. The data consists of general information about the situation based on the interview with the small ruminant owner, information about individual animals included in the sampling and their respective results for each tests. Kapa Test agreement was done for the result between IcELISA and RT-PCR diagnostic tests using SPSS. In addition, the study area sites were mapped using ArcGIS version 10.2.

4. RESULT

Due to the fact that this study was started in 2017, when only 44 samples were collected, the test conducted for them was only RT-PCR. The samples tested positive for RT-PCR (n=5) were kept at -80^{o}c. In 2018, additional 16 samples were collected and by adding the previous year's positive samples (for RT-PCR) a total of 21 samples were cultured and were also tested with IcELISA and RT-PCR.

Table 5: Summary of diagnostic tests used

No.	Type of Test used	Number of samples tested
1	Cell culture	8
2	IcELISA	21
3	RT-PCR	60

4.1. Observation on clinical signs of PPR

Clinical signs of PPR were observed in both sheep and goats, in both sex and all age groups, with a great variation in susceptibility in species (caprines are seen to suffer more), sex (male animals are prone) and age (younger animals are prone). The clinical signs included high fever, ocular and nasal discharge, respiratory distress and profuse diarrhea (Fig. 6).

a) b)

Figure 6: Pictures of sick animals displaying various clinical signs of PPR: a) serous nasal discharge and b) profuse diarrhea (picture taken from Daweharawa District)

4.2. Virus detection and confirmation

4.2.1. Immunocapture enzyme-linked immunosorbent assay (icELISA) result

Out of the total 60 samples, 21 samples were tested using immunocapture enzyme-linked immunosorbent assay (icELISA) for the direct detection of antigen, the PPR Virus. From the 21 samples tested, 18 were found to be positive for the test which counts for 85.7% of the tested samples. The samples were collected from five outbreaks and the antigen detection rate for each outbreak was different. For two of the outbreaks; BelojiGanfure of B/Gumuz (n=5) and Konso special District of SNNPR (n=10), all samples were positive for the test, thus the antigen detection rate was 100%.

Table 6: Antigen detection rate by Outbreak incidence

Region And District	Number of total sample	IcELISA result	
B/Gumuz, Belojiganfure	5	Positive 5 (100%)	
		Sex	Species
		Male 3	Sheep 0
		Female 2	Goat 5
Afar, Chifra	2	Positive 1 (% 50)	
		Sex	Species
		Male 1	Sheep 0
		Female 1	Goat 2
SNNPR, Konso	10	Positive 10 (100 %)	
		Sex	Species
		Male 4	Sheep 4
		Female 6	Goat 6
Amhara, Habru	1	Positive 0 (0 %)	
		Sex	Species
		Male 0	Sheep 0
		Female 1	Goat 1
Tigray, Enderta	3	Positive 2 (73%)	
		Sex	Species
		Male 1	Sheep 0
		Female 2	Goat 3
Total	21	Positive 18 (85.7 %)	
		Sex	Species
		Male 9	Sheep 4
		Female 12	Goat 17

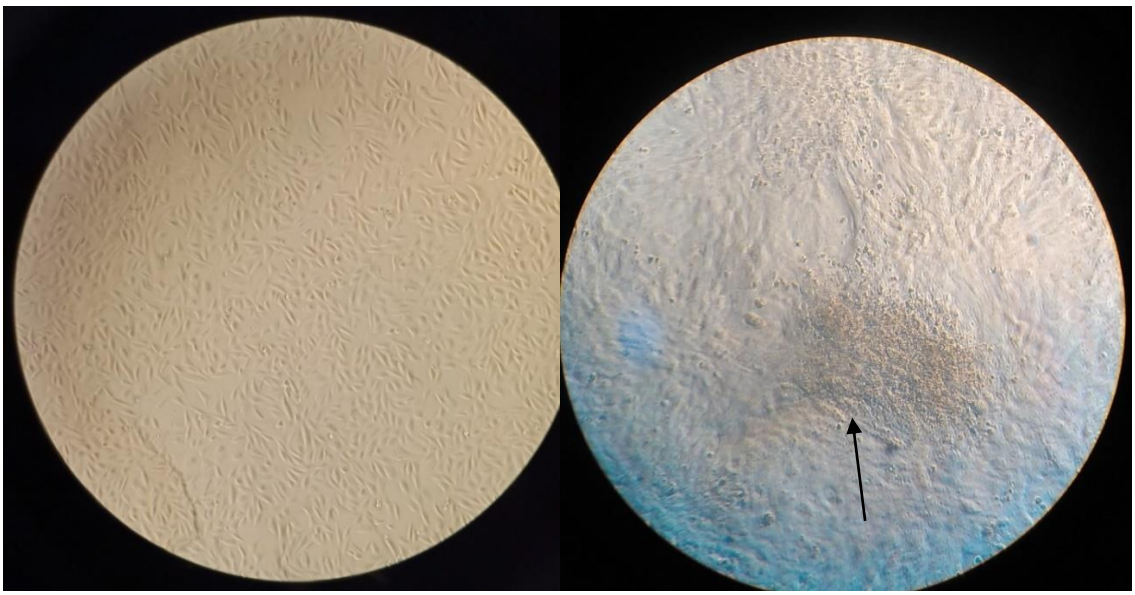
Followed by Tigray-Enderta (73%), Afar-Chifra (50%) and Amhara-Habru (0%) (Table 6). Among the cases tested using IcELISA (n=21); 18 were positive and among this, 10 (55.5%) were females, whereas the rest 8 (44.4%) were male. Age wise, detection rate in the young was 12 (66.6%), where the adult only holds 6 (33.3%). Species wise, sheep was only 4 (22.3%) where goats holding 14 (77.7%), (Table 7).

Table 7: Rate of detection of different variables

Total	SEX	AGE	Species
N= 21	Positive (N = 18)	Positive (N = 18)	Positive (N = 18)
	Male 8 (44.4%)	Young 12 (66.6%)	Sheep 4 (22.3%)
	Female 10 (55.5%)	Adult 6 (33.3%)	Goat 14 (77.7%)
	Negative (N = 3)	Negative (N = 3)	Negative (N= 3)
	Male 1	Young 3	Sheep 0
	Female 2	Adult 0	Goat 3

4.2.2. Cell culture

During this study 60 samples were collected from 9 suspected outbreaks. From these, 8 samples from two outbreaks were cultured for virus isolation. Viruses were isolated from all cultured samples. Isolation was evident by development of visible cytopathic effect of different degree on Vero cell lines and confirmed by ELISA .The growth of the virus was characterized by fusion of the cells, with the nuclei of the fused cells surrounding a granular area in the cytoplasm giving it a grape like appearance (Figure 6)



(a) (b)

Figure 7: Picture showing (a) Uninfected mono layer of Vero cells (b) Vero cells infected by PPRV showing a cytopathic effect (CPE)

4.2.3. PPRV detection using RT-PCR

All the 60 swab samples were tested using RT-PCR technique and only 8(13.4%) samples were positive. Highest detection rate of PPR virus nucleic acid was from Konso samples (n=10) where 5 (50%) of the samples were found positive, followed by Belojigamfure District (2/10), Habru (1/1) , Dawaharawa District (0/7) ,Telalak (0/2), Hadigala (0/18) and Jigjiga (0/7). The detection rate for each district is described in Table 8

Table 8: Result of RT-PCR for detection of PPRV nucleic acid in suspected field samples

District	RT-PCR detection rate in %	
	N	**Positive**
Belojigamfure	10	2 (20%)
Daweharawa	7	0 (0%)
Telalak	2	0
Hadigala	18	0
Jigjiga	7	0
Konso	10	5(50%)
Habru	1	1 (100%)
Chifra	2	0
Enderta	3	0
Total	**60**	**8 (13.4%)**

Among the cases tested using RT-PCR (n=60); 8 were positive and among this, 3 (37.5 %) were females, whereas the rest 5 (62.5%) were male. Age wise, detection rate in the young was 6 (75%), where the adult only holds 2 (25%). Species wise, sheep was only 2 (25%), where goats holding 6 (75%), (Table 9).

Table 9: Detection rate of RT-PCR in different factors

Total	**SEX**	**Age**	**Species**
N= 60	Positive (N = 8)	Positive (N = 8)	Positive (N = 8)
	Male 5 (62.5%)	Young 6 (75%)	Sheep 2 (25%)
	Female 3 (37.5 %)	Adult 2 (25%)	Goat 6 (75%)

Figure 8 shows the photograph of the gel electrophoresis of the PCR products that was analyzed. The fragment size of the amplified products was 351 bp as reported by Couacy-Hymann *et al.*, 2002).

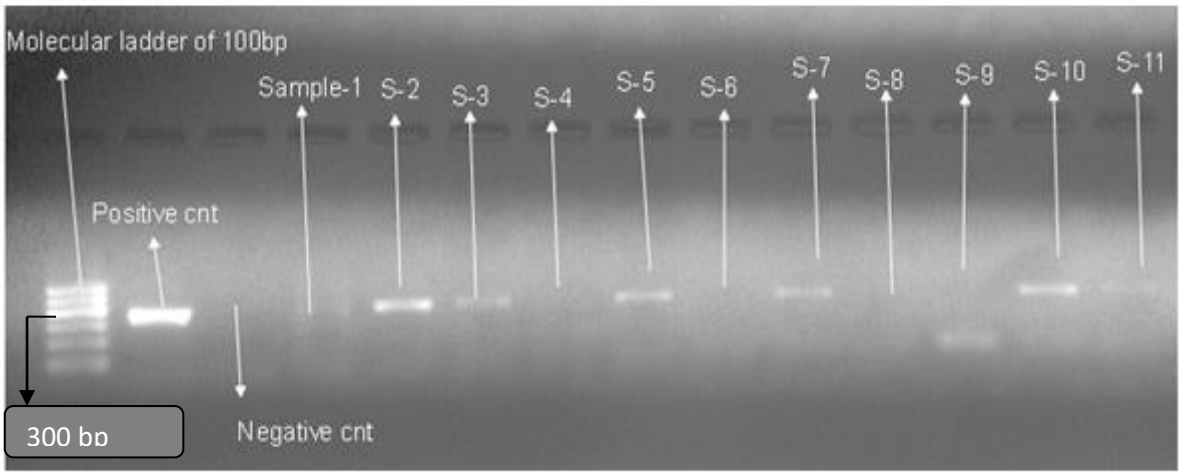


Figure 8: Picture of Agarose gel electrophoresis result of PCR products

4.2.4. Sequencing result

Unfortunately, the data obtained from the LGS genomics could not be analyzed and produce a meaningful result as indicated below in Figure 9.

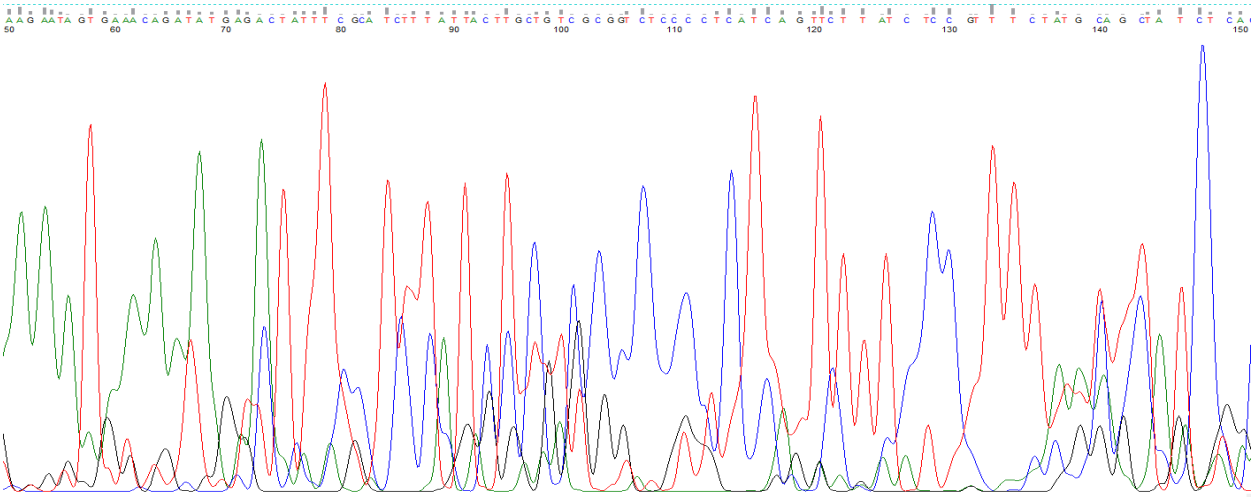


Figure 9: The result of data sequencing showing faint graphs due to low concentration

4.3. Test agreement of Ic-ELISA and RT-PCR results

A total of 60 samples were tested with RT-PCR technique and 21 samples out of the 60 were sued for Ic- ELISA. From the 21 samples both tests were conducted on, only 8 of them were positive for RT-PCR technique and 18 of them were positive for Ic-ELISA. The test agreement between RT-PCR and IcELISA was found to be 0.023, which is a low-test agreement (Table 10).

Table 10: Test agreement of Ic-ELISA and RT-PCR techniques

RT-PCR * IcELISA Cross tabulation

		RT- PCR		
		Negative	Positive	Total
IcELISA	Negative	2	1	3
	Positive	11	7	18
Total		13	8	21

5. DISCUSSION

In Ethiopia, studies on PPRV characterization are very few compared despite impact of the disease on the livelihood of pastorals and agro pastorals throughout the country. Since the first introduction of the disease, a couple of decades ago, much could have been done to control its spread throughout the regions, there by sparing thousands of small ruminants dying from the diseases every year. This would have added value to the day to day life of farmers and pastorals who are solely dependent on their livestock for their livelihoods. This study was undertaken in some of the areas which had a PPR outbreak incidence in small ruminants in the lowland areas of the country; Somali, Amhara, Afar, Tigray, SNNPR and B/Gumuz regional states of Ethiopia.

The present study confirmed PPRV exist in the sheep and goat populations in five of the nine studied districts ranging from lowland to mid-land agro-ecology, and that it has been more recently circulating in village flocks based on the pen-side test results.

The investigated flocks were tested using the pen-side test kit and 8 of the tested flocks were positive, but in the laboratory diagnosis using Ic-ELISA and RT-PCR techniques, only five of the outbreaks were positive. This could be due to lack of cold chain maintenance while transportation together with the fact that RNA viruses being very fragile. However, according to Abraham (2005) the PCR technique has proved invaluable for analysis of such poorly preserved field samples.

Host preference and transmission

In both tests, antigen detection rate was higher for goats; 6 (75%) in RT-PCR and 14 (77.7%) in IcELISA tests. This is in agreement with Wondimagegn (2014) where goats were found to have higher prevalence compared with sheep though the difference was not significant and Biruk (2014) also identified a significant higher rate of infection in goat than sheep samples with both Ic-ELISA and RT-PCR. The antigen detection rate is in agreement with a study made in Sudan where values in each species were 33.6, 21.1, 15.4, and 12.3% in camel, goat, sheep, and cattle, respectively (Intisar, 2017).

In the contrary, Abraham *et al*. (2005) found a higher PPR prevalence in sheep than goats. The differences observed in different studies could be due to the difference in the proportion of sampled animals. Additionally, goats are used for meat and milk compared with sheep, thus, pastorals keep more goats than sheep. Moreover, According to Biruk (2014) higher seroprevalence in goats than in sheep could be linked to higher productiveness in goats compared to sheep. It was suggested that new born kids account for a large proportion of the goat flock each year, which increase the size of susceptible population (Kihu *et al*., 2016; Megersa *et al.*,2012).

In this study, the antigen detection rate was higher in young animals with the age of less than one year \ 6 (75%) in RT-PCR and 12 (66.6%) in IcELISA, which is in agreement with previous studies stating young animals are less likely to have developed protective antibody titers and, therefore, are more susceptible to PPRV (Waret-Szkuta *et al*., 2008; Munir *et al.*, 2013). In contrast to this study, Biruk (2014) found the highest prevalence of PPR being observed in adults compared to young age in sero-surveillance study. This could be due to the severity of the disease in younger animals that they died in few days after contracting the virus. Besides, sick kids are usually left at home to get additional support and treatment provided by the owners.

In this study there was no much difference between sexes although the result was not confirmed by statistical test, 5 (62.5%) and 3 (37.5 %) in RT-PCR were male and Female respectively and 10 (55.5%) and (44.4%) were female and male respectively in IcELISA. Positive results were identified from both female and male cases, which shows a less difference in host preference of the virus. In contrary with this finding, studies in Bangladesh have shown that male goats are significantly more prone to PPR than females (Abdalla *et al.,* 2012; Johar, 2015). This could be due to the fact that adult males' turnover is higher because they are sold once they got the desirable body condition for the market need. Females are kept for production and thus, the flock composition will be stocked with more young age animals; this together with the age factor made the young male population at higher risk than the rest population in the flock. In another study, Wondimagegn (2014) reported that serologically female sheep and goats were 2.5 times

more exposed than male since females stay longer in the flock and once they are infected they could be easily detected by serological methods (Muhammad *et al.*,2012).

From the investigated nine outbreaks in nine districts, only three were positive for RT-PCR test; highest presence of PPR virus was recorded in Konso District (50%) followed by Belojigamfure (20%) showing a clear difference between regions and districts. This shows that there is a great variation on the burden of the circulating virus. Similar difference was recording in Tanzania where positivity in goat flocks varied between different study (Mvomero and Ngorongoro districts).

Diagnosis

In this study, three kind of antigen and nucleic acid detection methods were implemented. The first being the cell culture procedure. The cultured samples were grown till the second passage where clear cytopathic effect of the virus was detected. The appearance of cytopathic effects (CPE) may require at least 8-10 days or several blind passages. In Vero cells, the cytopathic effects (CPE) produced by PPRV consist of cell rounding, clumping into typical grape-like clusters, formation of small syncytia and appearance of long fine often anastomosing "spindle cells" (Abraham, 2005). However, not all the cultured cell fully grew, this could be due to most of the samples were nasal and ocular discharges. However, whatever the qualities of all the new techniques is, it is important to know though not necessarily used for all outbreaks, that virus isolation still remains the gold standard diagnostic technique (Balamurugan *et al.,* 2014).

The second test method used in this study was the IcELISA. This technique yielded more positive results (21 tested, 18 were found to be positive for the test which counts for 85.7% of the tested samples) other than the rest techniques implemented in this study. This is in agreement with Abraham (2005), who reported that immunocapture ELISA was found to be highly sensitive in detection of antigen in tissues and secretions of infected goats. It utilizes MAb directed against the nucleocapsid protein.

The third technique used was RT-PCR. This technique yielded the least number of positive result as compared to IcELISA and cell culture; only 8 positive results were found out of the total 60 samples. This could be due to different reasons such as the nature of the virus being RNA virus, and the type of samples taken for the study. This is in agreement with Kgotlele his colleagues, (2014). The rate of detection of PPRV was higher in tissues than in buffy coat, and ocular and nasal swabs. PPRV is routinely diagnosed on the basis of case history, geographic location, clinical examination, gross pathology and histological findings but confirmatory diagnosis is by conventional reverse transcription polymerase chain reaction (RT-PCR) (Kula, 2014). The legendary sensitivity of PCR combined with its ability to amplify specific sequences when specifically designed deoxyoligonucleotide primers are used, make it an attractive technique with which to detect viral RNAs (Brian and Hillar, 1996).

Over all, the difference in the field diagnostic method results i.e. the Pen-side test and the IcELISA could be attributed to many factors. After the pen-side confirmation of flocks, samples were collected with an interval of 1st week to 4th week of the outbreak occurrence date, which may lead to reduction of the level of active cases in the flock there by decreasing the chance of getting positive result. In addition, animals discharge active antigen in the first ten days of the disease cycle, and afterwards they could develop antibodies. As observed on the result, the earliest the investigation is done, the higher the chance of detecting the virus with IcELISA. On this study, the investigation done on the first week of confirmation was 60% of the positive results, and the investigation on the 2nd week was only 40% of the positive outbreaks and the rest investigations made on the 3rd and 4th week were totally negative.

The test agreement result between IcELISA and RT-PCR is very low. This could be due to the nature of the tests, where IcELISA is mainly relying on the usage of two monoclonal antibodies (MAb) raised to the N protein, which allows rapid identification of PPRV (OIE, 2013). Whereas RT-PCR depends on the extraction of RNA, making it more complex; hence the detection level reduces.

6. CONCLUSION AND RECOMMENDATION

The current study provides a glimpse on the circulation of PPRV in different districts of six regional states and stresses the fact that PPR is still among the major threat for the wide populations of small ruminants Ethiopia have. Among the nine outbreaks investigated, eight were pen-side positive and among this Five were IcELISA positive and two were RT-PCR positive, which pointed out that there is a great variation in the virus circulation in the flocks between the districts and even the regions. Further characterization of the study findings will help in the better understanding of the diseases situation.

Regarding diagnosis, the rapid detection by suitable and appropriate methods like the pen-side test could help in early detection of PPRV in infected flocks and will help in early diagnosis of infection, thereby facilitating the control of PPR disease in the country. Additionally, Characterization of the linages circulation in the country helps in better understanding of the disease, there by supporting the eradication program being implemented in the country. Focus should be given to strengthening the animal diseases reporting system to ensure the rapid response required while detection of positive flocks, thereby to contain the disease from further spread.

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8. ANNEX

ANNEX 1: Procedure of cell culture

- Prepare the hood, bring all the reagents and materials required to the hood
- Examine the cultures carefully for signs of deterioration or contamination
- Take the culture flasks to a sterile work area, remove and discard the medium
- Add PBS prewash (0.2ml/cm^2) to the side of the flasks opposite the cells so as to avoid dislodging cells, rinse the prewash over the cells, and discard. This step is designed to remove traces of serum that would inhibit the action of trypsin.
- Add trypsin (0.1ml/cm^2) to the side of the flasks opposite the cells. Turn the flasks over and lay them down. Ensure that the monolayer is completely covered. Leave the flasks stationary for 15-30seconds.
- Raise the flasks to remove the trypsin from the monolayer and quickly check that the monolayer is not detaching. Withdraw all but a few drops of the trypsin.
- Incubate, with the flasks lying flat, until the cells round up; when the bottle is tilted, the monolayer should slide down the surface.
- 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 so, 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. A single-cell suspension is desirable at subculture to ensure an accurate cell count and uniform growth on reseeding.
- Count the cells or follow the splitting ratio
- Dilute the cell suspensions to the appropriate seeding concentration and distribute among flasks.
- Incubate in 5% CO_2 at 39^0C.

ANNEX 2: Sample preparation and inoculation on mono layer cells

- Tissues are removed from the buffered glycerin or VTM (virus transport media)

- ❖ Put in a mortar and wash several times with PBS containing antibiotics and antimycotic
- ❖ by using coarse sterile sand, triturate thoroughly and a 10 % suspension is made in PBS with antibiotics and antimycotic
- ❖ the suspension is free thawed three times to facilitate the release of viruses from the tissues
- ❖ centrifuge at 3000 rpm for 10 minutes and the supernatant is collected and filtered using 0.22µ pore size filter before inoculation

ANNEX 3: IcELISA testing procedure

- Allow all reagents to come to room temperature ($21^{o}c$-$\pm5^{o}$) before use homogenize reagents by inversion or vortex.
- Add -25µl of dilution buffer 13 to all wells

  - 25µl of the negative control to wells A1 and B1
  -25µl of the positive control wells C1 and D1
  -25µl of each sample to be tested to the remaining wells
- agitate the plate for 2 min(1±min) at 2 $1^{o}c \pm 5^{o}c$
- cover the plate and incubate 45min±5min at $37^{o}c$ ($\pm 2^{o}$)
- wash each well 6 times with approximately 300µl of the wells between washing
- prepare the conjugate 1* by diluting the conjugate 10* to 1/10 in dilution buffer 19
- Add 100µl of the conjugate 1* to each well.
- cover the plate and incubate 30min ± 3min at 2 $1^{o}c \pm 5^{o}c$
- Wash each well 6 times with approximately 300µl of the wash solution. Avoid drying of the wells between washings.
- Add 100 µl of the stop solution to each well
- incubate 15 min ±2 min at 2 $1^{o}c \pm 5^{o}c$ in the dark
- Add 100 µl of the stop solution to ach well in order to stop the reaction
- read and record the O.D at 450 rpm

VALIDATION

The test is validated if

- The mean value of the positive control O.D (OD_{pc}) is greater than 0.500

  $OD_{pc} > 0.500$

- The ratio of the mean values of the positive and negative controls (OD_{pc} and OD_{NC}).is greater than 3
- $OD_{pc}/ OD_{NC} > 3$

ANNEX 4: Procedure of Extraction of RNA from swab specimens

- ❖ Vortex swab specimen fluid and transfer 500µl of sample into the microcentrifuge tube labeled with the specimen number.
- ❖ 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.
- ❖ 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.
- ❖ 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.
- ❖ 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)
- ❖ Add 500µl RPE buffer to the RNeasy column and centrifuge for 15 sec at >8000xg. Discard flow through from the collection tube.
- ❖ 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.
- ❖ Centrifuge the empty RNeasy column an extra 2 minutes at full speed and discard the collection tube.

- 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 -20oc or colder.

ANNEX 5: Procedure of RT-PCR

- Thaw all reagents, except reverse transcriptase and taq polymerase and possibly keep them on ice.
- Master mix preparation, Before preparing the reaction mix, it is necessary to calculate the correct volume of reagents to be used as in attached protocols.. Prepare a reaction mix as follow

RNase-free water (7.5µl)
5XPCR buffer (**5**µl)
DNTPs Mix 10mM (**1**µl)
Q solution (**5**µl)
Primer forward NP3 (**1.5**µl)
Primer reverse NP4 (**1.5**µl)
Qiagen enzyme mix (**1**µl)
RNA (**2.5**µl)

} Per sample

- Aliquate 22.5 µl of the prepared master mix in to approximately labeled 0.2microcentrifuge tubes in PCR work station for master mix
- Add 2.5 µl of RNA template in sample dispensing PCR work station and transfer them to the thermocycler
- Place the reaction tubes in the thermal cycler and setup temperature according to the protocol in the table below
- **Amplification cycle**

Steps	Temperature	Time	Cycle
1st	50 ºC	30 min	1 cycle
	95 ºC	15 min	
2nd	94 ºC	30 sec	40 cycles
	60 ºC	30 sec	
	72 ºC	1 min	
3rd	72 ºC	5 min	1 cycle
Put at	4 ºC	Till machine off	

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

ANNEX 6: Procedure of Agarose Gel Electrophoresis

- Prepare an adequate volume of 1xTBE-buffer to prepare the gel and to fill the electrophoresis tank
- 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.
- 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 55oc, add the required amount of Ethidium bromide in chemical hood. This prevents warping of the gel apparatus
- 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.

- 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.
- Apply 1ul 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.
- 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 Ethidium bromide (EtBr).
- Turn off the power supply when the bromophenol blue has merged a distance judged sufficient for separation of the DNA fragments.
- The DNA can be visualized on a UV transilluminatorand photographed