

Thesis Ref. No. _____

**EPIDEMIOLOGY AND IDENTIFICATION OF PESTE DES PETITS
RUMINANTS (PPR) VIRUS CIRCULATING IN SMALL RUMINANTS OF
EASTERN AMHARA REGION BORDERING AFAR, ETHIOPIA**

MSc Thesis



By

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Addis Ababa University, College of Veterinary Medicine and Agriculture, Department of
Veterinary Clinical Studies

June, 2014

College of Veterinary Medicine and Agriculture, Bishoftu

EPIDEMIOLOGY AND IDENTIFICATION OF PESTE DES PETITS RUMINANTS
(PPR) VIRUS CIRCULATING IN SMALL RUMINANTS OF EASTERN AMHARA
REGION BORDERING AFAR, ETHIOPIA



A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis
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Science in Tropical Veterinary Epidemiology

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College of Veterinary Medicine and Agriculture, Bishoftu

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by: **Biruk Alemu** entitled **Epidemiology and identification of Peste des Petits Ruminants (PPR) virus circulating in small ruminants of eastern Amhara region bordering Afar, Ethiopia** and recommend that it be accepted as fulfilling the thesis requirement for the degree of Masters of Tropical Veterinary Epidemiology.

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DEDICATION

This thesis manuscript is dedicated to my wife, Dr. Hayat Seid and our son, Yedideya Biruk who have provided me with emotional support, patience and encouragement.

STATEMENT OF AUTHOR

First, I declare that this thesis is my *bonafide* 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 an advanced (MSc) degree 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 degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

AGID	Agar Gel Immuno-Diffusion test
BoA	Bureau of Agriculture
cDNA	Complementary Deoxyribonucleic Acid
cELISA	Competitive Enzyme Linked Immunosorbent Assay
CHS	Contact Hypersensitivity
CIEP	Counter Immuno-Electrophoresis
CIRAD	Center for International Research and Agricultural Development
CPE	Cytopathic Effect
DIVA	Differentiate Infected from Vaccinated Animals
FAO	Food and Agricultural Organization
GIS	Geographical Information System
HA	Haemagglutination
HI	Haemagglutination Inhibition
HRP	Horse Radish Peroxidase
Ic-ELISA	Immunocapture Enzyme Linked Immunosorbent Assay
IFAT	<i>Indirect fluorescent antibody test</i>
MAb	Monoclonal Antibody
NP	Nucleoprotein
OD	Optical Density
OIE	World Animal Health Organization
PANVAC	Pan African Veterinary Vaccine Center
PAs	Peasant Associations
PCR	Polymerase Chain Reaction
PPRV	<i>Peste des petits ruminants Virus</i>
RNA	Ribonucleic Acid
RNP	RiboNucleocapsid Protein
RPV	Rinder Pest Virus
RT-PCR	Reverse Transcription- Polymerase Chain Reaction

LIST OF ABBREVIATIONS (Continued)

s-ELISA	Sandwich Enzyme Linked Immunosorbent Assay
SLAM	Signaling Lymphocytic Activation Molecules
TMB	Tetramethylbenzidine
UAE	United Arab Emirates
VNT	Virus Neutralization Test
VTM	Viral Transport Media

Table of Contents

STATEMENT OF AUTHOR	v
ACKNOWLEDGEMENTS	vi
LIST OF ABBREVIATIONS	viii
LIST OF TABLES	xii
LIST OF FIGURES	xiii
LIST OF APPENDICES	xiv
ABSTRACT.....	xv
1. INTRODUCTION.....	1
2. LITERATURE REVIEW	5
2.1. PPR: The Disease.....	5
2.2. The Virus Structure.....	6
2.3. Pathogenesis, pathogenicity and virulence	8
2.4. Epidemiological Situations	11
2.4.1. <i>Current geographic distribution</i>	11
2.4.2. <i>Quantifying the disease burden in various host demography, host range and reservoirs</i>	15
2.4.3. <i>Disease pattern and seasonal occurrence</i>	17
2.4.4. <i>Molecular epidemiology</i>	19
2.4.5. <i>Derivers (risk factors) for PPR transmission (challenging its eradication)</i>	21
2.5. Recent diagnostic methods	23
2.6. Progress and obstacles in vaccine development	27
2.7. Research needs.....	29
3. MATERIALS AND METHODS	31
3.1. Study area	31
3.2. Study population, sampling scheme and sample size	34
3.3. Study design and data collection.....	36
3.3.1. <i>Retrospective analysis of epidemiological data on PPR outbreak</i>	36
3.3.2. <i>Active field investigation and Questioner survey</i>	37
3.3.3. <i>Serological study</i>	38
3.3.4. <i>Antigenic detection using Immuno-capture ELISA (I_C-ELISA)</i>	39
3.3.5. <i>Molecular detection of the virus nucleic acid</i>	40
3.3.6. <i>Cell culture and virus isolation</i>	41

3.4.	Data management and analysis	42
4.	RESULTS	44
4.1.	Temporal and spatial distribution of PPR using 5 years retrospective data.....	44
4.1.1.	<i>Temporal distribution of PPR</i>	44
4.1.2.	<i>Spatial distribution of PPR</i>	45
4.2.	PPR clinical disease investigation through participatory approach	47
4.2.1.	<i>Community perception of PPR based on questionnaire interview</i>	47
4.2.2.	<i>Observation on clinical signs of PPR and quantifying its magnitude</i>	48
4.2.3.	<i>Pen-side test/ Lateral flow device</i>	51
4.3.	Serological studies	51
4.3.1.	<i>Seroprevalence of PPR and associated risk factors in unvaccinated population</i> ..	52
4.3.2.	<i>Seroprevalence and its assumed determinants in vaccinated population</i>	55
4.3.3.	<i>Determining the cut-off PI values of the cELISA</i>	56
4.4.	Virus detection by I _c -ELISA, isolation on CHS-20 cell lines and confirmation using RT-PCR, and IFAT assay	61
4.4.1.	<i>Virus detection using IcELISA</i>	61
4.4.2.	<i>Virus detection and confirmation using RT-PCR</i>	61
4.4.3.	<i>Virus detection, isolation on CHS-20 cell lines and confirmation on IFAT assay</i>	63
4.5.	Test agreement of RT-PCR and Ic-ELISA and cell culture results of pooled samples .	65
5.	DISCUSSION.....	67
5.1.	Spatio- temporal retrospective data analysis.....	67
5.2.	Epidemiological observations and disease investigation	68
5.3.	Seroprevalence of PPR virus antibodies and associated risk factors in unvaccinated population	69
5.4.	Seroprevalence of PPR virus antibodies /herd immunity in vaccinated animals	73
5.5.	Comparative protective antibody level of the vaccinated and unvaccinated population based on the PIs	75
5.6.	Detection of PPR virus in small ruminants.....	76
5.7.	Virus isolation and confirmation	78
6.	CONCLUSION AND RECOMMENDATIONS	79
7.	REFERENCES.....	82
8.	APENDICES	105

LIST OF TABLES

Table 1: Comparison of rinderpest full-length genome sequence with those of some other morbilliviruses	6
Table 2: Selected prevalence studies of PPR in different hosts in different countries and districts of Ethiopia	16
Table 3: Study areas, zonal sheep and goat population and respective number of sample	36
Table 4: The mortality, morbidity and CFR during PPR outbreak in Habru district in 2014	50
Table 5: Magnitude of seroprevalence levels among various vaccination statuses in small ruminants as compared among five Districts	52
Table 6: Prevalence of PPR antibodies in sera of small ruminants using cELISA	52
Table 7: Association of assumed exposure variables with seropositivity of PPR	54
Table 8: Multivariable Logistic regression analysis of variables associated with sero-positivity to PPR in non-vaccinated sheep and goats	55
Table 9: Association of assumed determinants/ fixed variables with sero-conversion of PPR antibody in vaccinated population	56
Table 10: Results of Ic-ELISA for detection of PPR antigen in suspected field samples	61
Table 11: Results of RT-PCR for detection of PPR viral nucleic acid in suspected field samples	63
Table 12: Species-wise detection of viral antigen and nucleic acid	63
Table 13: Test agreement of RT-PCR and Ic-ELISA and cell culture results of pooled samples	66

LIST OF FIGURES

Figure 1: Morbillivirus	7
Figure 2: Seroprevalence of PPR across wereda in Ethiopia	12
Figure 3: Worldwide cumulative distribution of the four PPR virus lineages.	21
Figure 4: Map of Ethiopia showing Amhara region, study districts and sampling areas	34
Figure 5: PPR seasonal disease pattern and case fatality rate during 2009-2013	44
Figure 6: Region wise number of outbreak during 2009-2013	45
Figure 7: Map showing PPR outbreak report status of Ethiopia (2009-2013)	46
Figure 8: Suspected clinical symptoms of PPR as reported by respondents	47
Figure 9: Various clinical signs of PPR	49
Figure 10: PPR pen-side test/ Lateral flow device	51
Figure 11: Distribution of % of colour inhibition for PPR antibody positive and negative samples by cELISA	57
Figure 12: Distribution of % of colour inhibition for vaccinated versus unvaccinated small ruminants along with cut-off value for detection of positive (PI \geq 50%) and viral challenge protective (PI \geq 76%) antibody titer in the test sera using cELISA	58
Figure 13: Prevalence of PPR antibody for positivity and protection in vaccinated versus unvaccinated PAs	59
Figure 14: Proportion of population (a) positive antibody titer for PPR (PI \geq 50%) and (b) protective antibody titer for viral challenge (PI \geq 76%) using cELISA in the vaccinated versus unvaccinated population	60
Figure 15: Agarose gel electrophoresis of PCR products (351 bp) amplified with NP3 and NP4, PPR specific primers.	62
Figure 16: Photographs of the microscopic observation of the CHS-20 cells	64
Figure 17: Detection of the PPRV H protein by immunofluorescence staining	65

LIST OF APPENDICES

Appendix I: Questionnaire	105
Appendix II: Procedure of Extraction of RNA from swab specimens using Qiagen® RNeasy Mini Kit	108
Appendix III: Procedure of RT-PCR	109
Appendix IV: Procedure of Agarose Gel Electrophoresis	110
Appendix V: Procedure of cell culture	111

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Agriculture

ABSTRACT

A cross-sectional study design was employed between November 2013 and April 2014 to determine antibody/immunity level of vaccinated sheep and goats at herd level, to determine the sero-prevalence and risk factors of PPR in non-vaccinated areas and to investigate incidence and recent circulation of PPR virus in field samples collected from small ruminants of eastern Amhara region bordering Afar, Ethiopia. A total of 969 serum samples were collected from sheep and goats in the study districts. Multistage sampling, with four hierarchical stages, was used as sampling strategy. Peasant Association /villages and individual animal were selected by random sampling. A total of 32 samples, comprising 18 swab samples and 14 heparinized whole blood samples were collected from the suspected animals for the presence of PPR viral antigen. 28 samples were examined for the presence of PPRV RNA by one step RT-PCR assays. Anti-mortem samples were collected from active cases with the aim of virus isolation. In addition five years retrospective data on PPR outbreak was used in the present study. Questionnaire survey was also conducted to assess the association of seroprevalence of PPR and possible risk factors. Proportions were calculated for seroprevalence visa-vis fixed factors that included animal species, sex and age, districts and village. Univariable analysis for the proportions was carried out using Chi-square analysis in Epi Info software. A confidence limit of less than 5% was used to indicate a significant level. All variables with $P < 0.05$ (two-sided) in the univariable analysis were further tested by multivariable logistic regression model to assess their effect on PPR seropositivity. Clinical signs of PPR were observed in both sheep and goats, in both sex and all age

groups, in the all study districts. The clinical signs included high fever, ocular and nasal discharge, few abortions, respiratory distress and diarrhea. The disease outbreak was severe in Habru district with morbidity, mortality and case fatality rates of 21.9%, 8.4% and 38.4 % in small ruminants, respectively. The overall seroprevalence of PPR virus antibody was 28.1%, 64.5% and 56.5% in unvaccinated, vaccinated and unknown vaccination status of small ruminants, respectively. The multivariable logistic regression model revealed residing in Rayakobo district, adult age, communal grazing and recent introduction of new animals as risk factors for PPR seropositivity in sheep and goats. There was statistically significant ($p= 0.000$) differences in the level of seroconversion among the vaccinated districts with similar vaccine response of different age groups, sex, and species involved. In unvaccinated population around 28.1% and only 24.5% were with $PI \geq 50\%$ and 76%, respectively, whereas in vaccinated population around 64.5% and 58.8% of animals were with $PI \geq 50\%$ and 76%, respectively. The median value of PI is 26 and 83 for unvaccinated and vaccinated population, respectively indicating the success of the vaccine administered as the population has gained protection with 57 additional value of PI due to vaccination. 31.3% and 46.4 % of clinical samples examined were positive with I_c -ELISA for PPR viral antigen and RT-PCR for viral nucleic acid, respectively. The species-wise disease outbreaks were more severe in goats than sheep with both tests. The PPR virus were also successfully isolated on CHS-20 cell lines and confirmed with RT-PCR and IFAT assay only from samples collected from Habru district. The clinical and molecular findings of this study confirmed the circulation of PPR virus among populations of sheep and goats in the study areas and prevalence in actual outbreaks situation, which should be kept in mind while deciding the vaccination strategy for the control of the disease. The restriction of movement of animals from endemic areas, with rigorous quarantine and surveillance procedures should be practiced to prevent the spread of the disease and the transmission of the virus to different localities.

Key words: PPRV, small ruminants, risk factors, herd immunity, Eastern Amhara

1. INTRODUCTION

Sheep and goats are vital livestock for supporting food security because of their high reproductive capacity; faster growth rates, greater environmental adaptability and low initial investment, and hence have a unique niche in smallholder agriculture (Tibbo, 2006). There is an immense opportunity for increased livestock production in Ethiopia with growing human population, urbanization, economic development, domestic and export markets. However, prevalence of different diseases is found to be a major constraint of the sector.

Peste des petits ruminants (PPR) is one of the diseases of major economic importance and imposes a significant constraint upon sheep and goat production owing to its high mortality rate. It is an acute, highly contagious and frequently fatal disease of sheep and goats caused by PPR virus (PPRV), a member of genus morbillivirus of family Paramyxoviridae (Zahur *et al.*, 2009).

PPR is widespread in Africa, Arabia, the Middle East and in some geographical areas of Asia, including much of the Indian subcontinent. Furthermore, because of outbreaks in Morocco and the existing commercial trade between Morocco and both Algeria and Spain, the situation raised huge concern owing to the increased risk of introduction of the disease into free zones in northern Africa and into Europe (FAO, 2009; Khalafalla *et al.*, 2010).

The disease is mostly present in developing countries which often rely heavily on subsistence farming of small ruminants for trade and food supply (De Nardi *et al.*, 2012). Since 2007, more than one billion small ruminants in Africa and Asia have been considered at risk of being infected with the PPRV (FAO, 2009). 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) (Albina *et al.*, 2013).

The most effective way to control PPR is mass immunization of small ruminants as often, farmers in areas where the virus is endemic are unable to afford and implement the strict sanitary control measures, including the stamping out policy, required to contain the virus. Therefore, the control of PPR requires an effective vaccine and for this purpose several vaccines including both homologous and recombinant vaccines have been developed (Abubakar *et al.*, 2011a).

The homologous vaccine, however, also has certain limitations, including requirement for cold-chain maintenance, and inability to differentiate vaccinated from infected animals (DIVA). Alternative thermotolerant PPR-recombinant poxvirus vaccines have been engineered in the past (Jones *et al.*, 1993; Diallo *et al.*, 2002, 2007; Berhe *et al.*, 2003; Chen *et al.*, 2010), which should provide simultaneous protection against both diseases, although none of them have yet been launched in the market and used in the field.

Development and technology transfer of an efficacious thermo-stable vaccine against PPR (and sheep and goat pox) in the short term remains an area for further research (FAO, 2013). Furthermore, a DIVA vaccine would facilitate surveillance for actual disease during an ongoing vaccination campaign. It is also essential to fully understand the role of wildlife in the spread and potential maintenance of PPRV in the environment in order to be able to initiate successful control strategies (Baron *et al.*, 2011).

Another major gap for the success of PPR control is the lack of economical assessment of control strategies, and even of PPR cost. Such information would be useful to help veterinary services in convincing governments and international organizations to support and fund PPR control (Albina *et al.*, 2013).

The disease was clinically suspected for the first time in Ethiopia in 1977 in a goat herd in the Afar region, in the east of the country. Clinical and serological evidence of its presence has been reported in 1984 and later 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).

In spite of the fact that PPR disease outbreaks are underreported, due to the poor reporting system in Ethiopia, an increasing trend has been observed in PPR outbreaks between the years 1996 and 2005. Over the last two decades PPR has spread from the now endemic lowland pastoral communities to many districts in the highlands of Ethiopia. As expected, that the prevalence was much higher in the lowland pastoral systems as compared to the highland sedentary systems (Waret-Szkuta *et al.*, 2008).

The relentless spread of the disease in affected countries and the subsequent threat imposed on PPR-free African countries further south of the current endemic area have resulted in an increased recognition of the urgent need to embark on controlling peste des petits ruminants (PPR). Ethiopia developed a strategy for the progressive control of PPR that builds upon the lessons learnt from rinderpest eradication. A progressive control campaign based on repeated inoculation of all susceptible small ruminants is unaffordable to be implemented. Hence, an epidemiologically based targeting of endemic populations and high-risk zones will be essential. Despite an expansion of PPR to previously unreported area, very little work exists in the country to clearly reveal the epidemiology of the disease (Abraham *et al.*, 1991; Abraham *et al.*, 2005; Waret-Szkuta *et al.*, 2008; Megersa *et al.*, 2011; Delil *et al.*, 2012). Therefore, additional epidemiological and socio-economic studies are needed to support the current Ethiopian initiative towards controlling the disease.

Hence, the objectives of the study were:

- To determine antibody/immunity level of vaccinated sheep and goats at herd level
- To determine the sero-prevalence and risk factors of PPR in non-vaccinated areas
- To investigate incidence and recent circulation of PPR virus in field samples

2. LITERATURE REVIEW

2.1. PPR: The Disease

Peste des petits ruminants (PPR), also known as goat plague, is a highly contagious and infectious viral disease affecting domestic and wild small ruminants (Furley *et al.*, 1987). It is caused by a virus which belongs to the *Morbillivirus* genus of family *Paramyxoviridae*: the Peste des Petits Ruminants Virus (PPRV). PPR got different designations in the past, which almost all refer to the clinical signs of the disease: erosive stomatitis, goat enteritis, catarrhal fever of goat and Kata (Nigerian local name that means catarrh in English). The term goat plague was also used due to the reason that the disease was producing high level of mortality in goat populations (Berhe, 2006).

Since its first description in Côte d'Ivoire in 1942, the disease has steadily progressed over time throughout across Africa, the Middle East, and Asia (Libeau *et al.*, 2014). The infection has long been considered as caused by a variant of rinderpest virus, adapted to small ruminants. The recognition of PPR virus as a novel member of the *Morbillivirus* genus occurred only in the late 70s by using more sensitive laboratory techniques (Gibbs *et al.*, 1979).

PPR covers three essential forms: a 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 (Roeder and Obi, 1999). 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 (Diallo, 2006). Abortions are often observed during PPR

outbreaks, caused by PPRV alone or in combination with other pathogens (Kulkarni *et al.*, 1996; Abubakar *et al.*, 2008).

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 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).

2.2. The Virus Structure

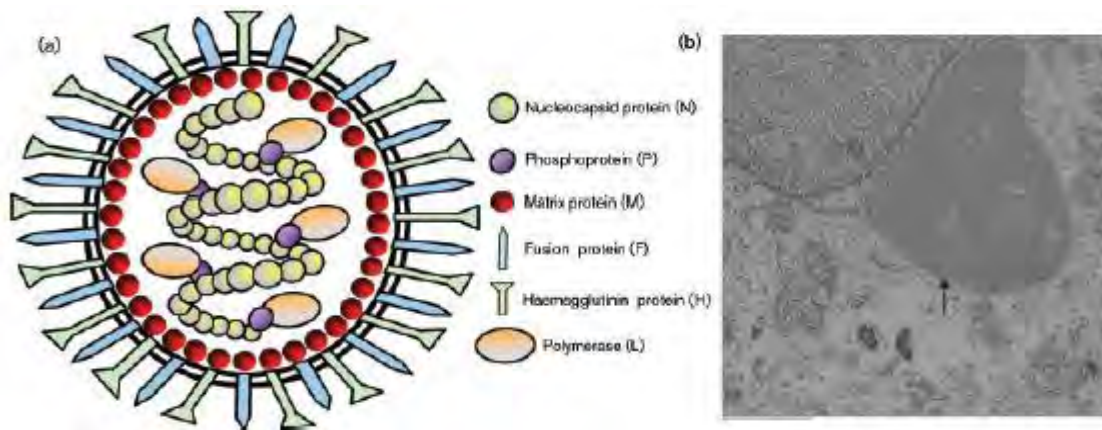
Like other members of the family Paramyxoviridae, PPR virus is an enveloped pleomorphic particle whose diameter varies between 150 and 700 nm, with a mean of 500 nm (Barrett, 1999). The virus envelope is 8-15 nm thick with glycoprotein spikes of 8.5-14.5 nm length being present throughout the membrane (Abubakar *et al.*, 2011a).

Table 1: Comparison of rinderpest full-length genome sequence with those of some other morbilliviruses

Virus	Genome length in nucleotides	Percentage of similarity with rinderpest
Rinderpest	15882	100
Measles virus	15894	70.50
Peste des Petitis	15948	66.98
Ruminantis		
Dolphin Morbillivirus	15882	65.91
Canine Distemper	15702	63.58

Source: Diallo *et al.*, (2007)

The genome of PPRV is non-segmented, single stranded RNA, approximately 16kb long with negative polarity (Haas *et al.*, 1995). It is composed of 15,948 nucleotides, the longest of all morbillivirus genomes sequenced so far (Diallo *et al.*, 2007) (see Table 1). The genome encodes eight proteins including six structural proteins namely: the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the haemagglutinin protein (H) and the large polymerase protein (L), and two nonstructural proteins V and C; in the order 3'-N-P(C/V)-M-F-H-L-5' (Bailey *et al.*, 2005) (See Fig. 1.).



Source: Banyard *et al.*, 2010

Fig. 1: Morbillivirus. (a) A schematic diagram of morbillivirus virion structure. (b) electron micrograph of viral ribonucleoprotein (RNP) present within an RPV-infected cell. The RNP is clearly 'herring-bone' structure (arrow) and is known to contain viral RNA and proteins within the cytoplasm, Bar, 2000nm.

The N protein totally encapsidates the viral RNA and encapsidated genomes can be observed under the electron microscope as 'herringbone-like' structures of approximately 14-23 nm in length (Barrett *et al.*, 1993). These structures containing the genomic RNA, completely encapsidated in N protein, and in association with the P and L proteins, form the minimal replicative unit of these viruses, the ribonucleocapsid protein (RNP) complex. The P protein acts as a molecular intermediate that is proposed to bridge components of the RNP during both the replicase and transcriptase activities of the viral life cycle (Abubakar *et al.*, 2011a).

The viral envelop, which derives from the host cell membrane, are associated three viral proteins: the matrix protein (M) which is located inside the envelope and serves as a link between the nucleocapsid and the two external viral proteins, the fusion protein (F) and the haemagglutinin (H), is seen as peplomers protruding from the envelope and believed to be important for virus particle assembly (Diallo *et al.*, 2007). By this position, M plays an important role in ensuring efficient incorporation of nucleocapsids into virions during the virus budding process. The haemagglutination allows the virus to bind to the cell receptor during the first step of the viral infection process. By their positions and their functions, both F and H are very important for the induction of protective host immune response against the virus (Diallo *et al.*, 2002; Berhe *et al.*, 2003).

The virus binds to the host cell receptor through H during the first step of the infection process. Following attachment, F mediates the fusion of the viral envelope with the host cell membrane, introducing the viral RNP complex into the cell cytoplasm. F and in particular, H are considered to be very important for inducing protective host immune response against the virus (Barrett *et al.*, 2005).

However N the most abundant and also the most immunogenic among PPRV proteins, does not induce protective immunity against the virus. It has been used in the development of diagnostic tests (Diallo *et al.*, 2007).

2.3. Pathogenesis, pathogenicity and virulence

PPRV is lymphotropic and epitheliotropic and produces severe lesions in organ systems rich in lymphoid and epithelial tissues. After entry through the respiratory route, PPRV multiplies in the pharyngeal and mandibular lymph nodes and tonsils (Munir *et al.*, 2013). The febrile stage may occur on the fifth day and may persist until the sixteenth day post-infection. The resultant viraemia facilitates the dissemination of the virus to all visceral lymph nodes, bone marrow, spleen, and the mucous membranes of both the

respiratory and digestive tracts (Abubakar *et al.*, 2011a) . Acute disease is usually accompanied by lymphopenia and immuno-suppression, leading to secondary opportunistic infections (Appel and Summers, 1995; Murphy and Parks, 1999). The virus can be isolated from nasal discharges from the day ninth of virus infection. PPRV then starts multiplying in the gastrointestinal tract, which leads to stomatitis and diarrhea.

Studies have showed that the apoptosis of infected cells seems to play an important role in the pathogenesis of PPRV in goats and sheep (Mondal *et al.*, 2001; Guvenc and Gurcan, 2009). Virus spread to oral lesions has been reported in several studies (Brindha *et al.*, 2001; Gulyaz and Ozkul, 2005). Al-Naeem and Abu-Elzein (2008) demonstrated the presence of viral antigen in papules around the oral cavity, which is an indication of the predilection site for viral replication and tropism like the measles virus, a skin lesion-causing virus in humans (Al-Naeem and Abu-Elzein 2008). Although this prediction is helpful to understand the pathogenesis of the disease, further studies are required to confirm that this is not due to other concurrent infections.

PPR virus exhibits different levels of virulence between sheep and goats. PPRV is sometimes reported as being more severe in goats than in sheep. Extensive species based antibody surveys have indicated that the level of antibodies against the PPRV N protein was higher in sheep than in goats. This explains why the virus might have more affinity in goats than sheep. Wosu (1994) has observed that the rate of recovery is lower in goats than in sheep (Wosu 1994). The difference in pathogenicity between sheep and goats may not be due to viral affinity, but may be due to a high recovery rate in sheep.

However, there have been reports of both an increased susceptibility of sheep populations, goat populations and outbreaks that appear to have affected sheep and goat populations equally (Taylor *et al.*, 2002; Singh *et al.*, 2004a; Yesilbag *et al.*, 2005; Chauhan *et al.*, 2009; Wang *et al.*, 2009). Asymptomatic infections are also known to occur in some species (Bidjeh *et al.*, 1995) although how the virus circulates in the

absence of clinical disease is not understood. PPRV is known to infect cattle asymptotically (Diallo *et al.*, 1989; Anderson and McKay, 1994) and can also affect camels (Roger *et al.*, 2001).

An outbreak with a high mortality in sheep was reported by Taylor (1984) who hypothesized that sheep possessed an innate resistance to the clinical effects of disease, but occasional field strains could overcome this resistance and produce high mortality (Taylor, 1984).

Breed may affect the outcome of PPR virus. Virulence appears to vary from strain to strain, although there is only one serotype. Different strains of PPRV have been shown to exhibit varied virulence when experimentally infected into the same breed of goat (Couacy-Hymann *et al.*, 2007b), and different breeds of goat have been shown to respond differently to infection with the same virus (Diop *et al.*, 2005). The reason for this variability is unclear but both virus sequence and host species are thought to be of importance.

Morbidity and mortality rates are often higher in young animals than in adults. Whilst disease can be severe, different animals respond differently following infection in both natural and experimental settings (Diop *et al.*, 2005; Couacy-Hymann *et al.*, 2007a, b). Prerequisites for susceptibility remain unknown but the infecting strain, immunological competence, nutritional status and pre-existing parasitic infections are all thought to contribute to the outcome of infection (Couacy-Hymann *et al.*, 2007b).

2.4. Epidemiological Situations

2.4.1. *Current geographic distribution*

PPR was long considered to be confined to West Africa but later it has expanded to cover large regions of Africa, the Middle East and Asia (Khan *et al.*, 2008). The chronological spread of PPRV, as recorded by detection in previously unaffected countries, gives the impression that the geographical spread of PPR occurred eastward, from West Africa to Bangladesh. However, this does not necessarily mean that PPR originated in West Africa (Arzt *et al.*, 2010). The global spread of PPR is probably related to the progressive control and later, eradication of rinderpest. The cessation of rinderpest vaccination campaigns and loss of antibody cross-protection between the two diseases means that small ruminants are now fully exposed to PPR (Libeau *et al.*, 2011). Since the middle of the 1980's, the development of new tools for diagnosis, allowing for a specific identification of the virus, has helped in determining the area of distribution of PPR (EMPRES WATCH, 2008).

In Africa, PPR endemic zones include the countries located between the Sahara and the Equator, from the Atlantic Ocean to the Red Sea. The disease is widespread in western, central, eastern and northern Africa (Banyard *et al.*, 2010; OIE, 2011a), and the four genetic lineages are all present in different regions of the continent.

In northern Africa, apart from Egypt which is infected at least since 1989 (Ismail and House, 1990) and more recently an outbreak in 2006 in Aswan province (El-Hakim, 2006), the Moroccan outbreak in 2008 was the first PPR incursion. PPR has now been identified in Tunisia (Ayari-Fakhfakh *et al.*, 2011) and Algeria (De Nardi *et al.*, 2012). It has now spread north of the Sahara and only Libya has not reported the disease to date in the region (Banyard *et al.*, 2010; OIE, 2011b).

In East Africa, confirmation of endemicity of PPRV has been shown through the detection of antibodies to PPRV in Kenya (2009), Uganda (2005 and 2007) and Tanzania (Swai *et al.*, 2009). PPRV was recently detected in Kenya in 2006 in the Turkana district (Anonymous, 2008). Somalia was also affected by PPRV in 2006 with the central regions being most seriously affected. Whilst not confirmed by laboratory diagnosis, PPRV was also seen in Ethiopia in 2008 and 2009 (Nyamweya *et al.*, 2009). In a study carried out in Ethiopia, analysis of national serological data revealed the seroprevalence and distribution of the disease in the country as shown in Fig. 2 (Waret-Szkuta *et al.*, 2008).

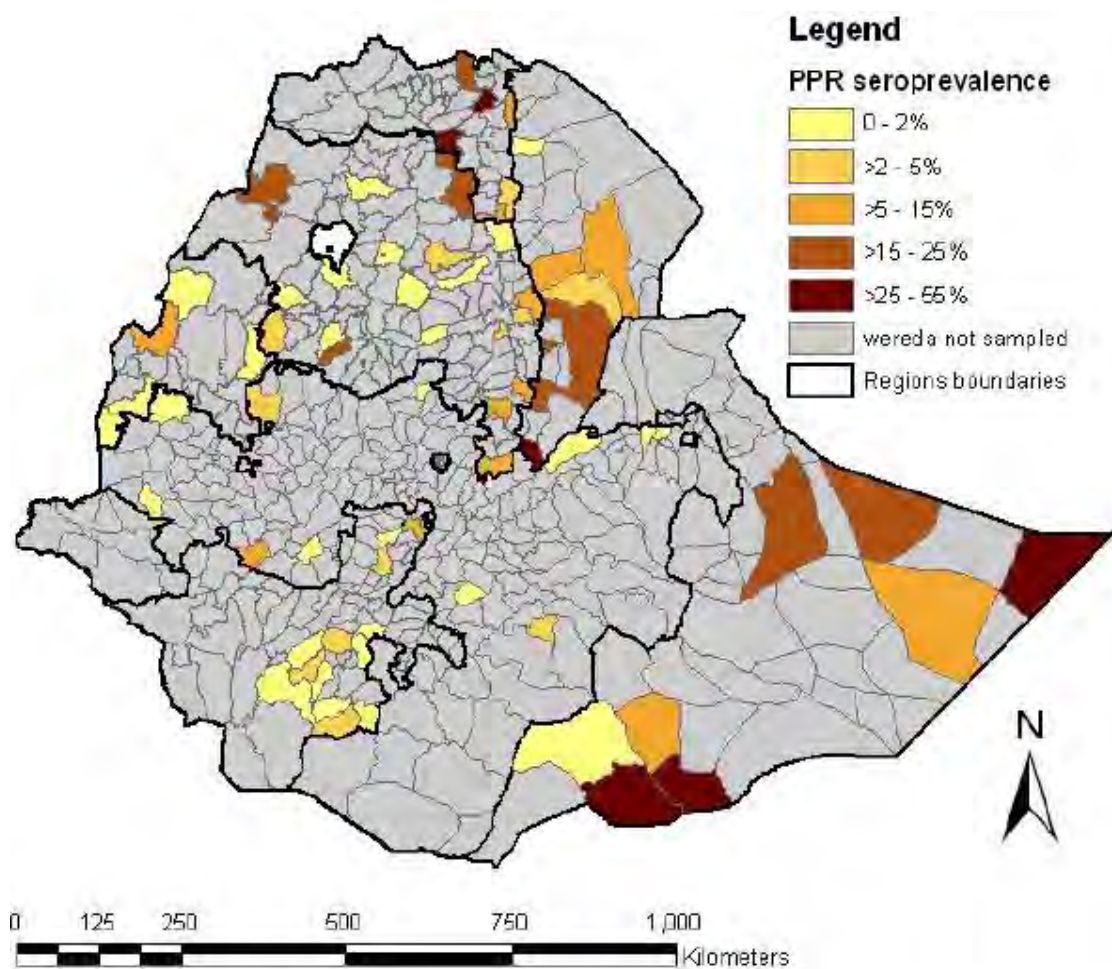


Fig. 2: Seroprevalence of PPR across woreda in Ethiopia. Administrative map of Ethiopia indicating the regions and woredas boundaries. For each woreda seroprevalence of PPR was calculated by dividing the number of positive valid samples by the number of individual sampled in the woreda. As the colour gets browner higher is the seroprevalence found in the area. In grey, woreda for which no data was available. (Source: Waret-Szkuta *et al.*, 2008)

In recent years, field data and laboratory findings have confirmed the dramatic spread of PPR toward the central and south of Africa, affecting Central Africa Republic (2005 and 2006), Democratic Republic of Congo (2006), Chad (1999 and 2006), Cameroon (2009) and Gabon (2007) (Banyard *et al.*, 2010). In October 2012, PPR has been reported for the first time in Angola (OIE notification). The risk of PPR introduction is now high for neighboring countries with major sheep and goat populations like Mozambique or Zambia (Albina *et al.*, 2013).

Furthermore, the presence of the either antibodies to the virus or the detection of viral nucleic acid in samples from Burkina Faso (2008), Ghana (2010), Nigeria (2007) and Senegal (2010) has been confirmed (Banyard *et al.*, 2010).

In Asia, PPR was first discovered in southern India in 1987. Subsequently, epizootic PPR spread across the Arabian Peninsula, the Middle (Housawi *et al.*, 2004; Al-Dubaib, 2008; Abu-Elzein *et al.*, 2004; El-Rahim *et al.*, 2005) and Far East, and several Asian countries like China, which has reported its first case of PPR in Tibet (Wang *et al.*, 2009) and in Nepal in 2007.

The virus continues to be reported periodically across India with recent reports documenting presence of PPRV in Rajasthan in the north (Kataria *et al.*, 2007), the Kolkata region in the east (Saha *et al.*, 2005), Karnataka and Maharastra in the southwest (Chavran *et al.*, 2009; Santhosh *et al.*, 2009) and across the southern peninsula (Raghavendra *et al.*, 2008). These reports reflect the endemicity of the disease across the entire country.

On the remainder of the Arabian Peninsula, the virus has been detected in a game reserve in the United Arab Emirates (Kinne *et al.*, 2010) as well as in Qatar (2010). Tajikistan reported their first cases of PPR. In Pakistan, serum samples from healthy animals in a goat flock following a suspected outbreak in 2005 were seropositive for PPRV antibodies

(Ahmad *et al.*, 2005) with further reports in the north of the country (Abubakar *et al.*, 2008; Mehmood *et al.*, 2009), north, south and central Punjab (Durrani *et al.*, 2010), Lahore (Rashid *et al.*, 2008) and in Islamabad where an outbreak in Afghan sheep (Bulkhi) occurred (Zahur *et al.*, 2009).

Further episodes of PPRV disease in Iran are reviewed by Abdollahpour *et al.*, (2006). PPRV is also believed to be present in Kazakhstan although only very few seropositive animals have been identified (Lundervold *et al.*, 2004). Serological detection of antibodies to PPRV has also been detected in samples from Vietnam (Maillard *et al.*, 2008).

After the first report of PPRV in European Turkey in 1996, the potential for PPRV to spread across the rest of Europe became the question of interest (Ozkul *et al.*, 2002). Indeed, there have been numerous reports of PPRV in Turkey having now also been reported in Western Turkey, Bursa province (Yesilbag *et al.*, 2005) and Mugla and Aydin provinces (Toplu, 2004) in the Aegean district.

In 2007, the first outbreak of fatal PPRV was reported in Kirikkale Province, Central Anatolia (Kul *et al.*, 2007), suggesting spread of the virus into the central belt of the country and in 2009 infection of sheep in the Middle and Eastern Black sea region of Turkey was reported (Albayrak and Alkan, 2009). The Moroccan outbreak and the potential existence of as yet- unidentified foci of PPRV infection across other territories in northern Africa also increases the threat of movement of infected animals into southern Europe. Historical exchanges exist between Morocco and Spain where both ovine and caprine populations are important.

2.4.2. Quantifying the disease burden in various host demography, host range and reservoirs

PPRV primarily infects sheep and goats, although cattle, buffaloes, camels and pigs are susceptible to infection, but do not contribute to the epidemiology as they are unable to excrete virus (Banyard *et al.*, 2010). Cattle and pigs are known to be a dead end host and all attempt to induce clinical disease in adult cattle experimentally failed (Abraham, 2005). Whether PPRV-infected and sick buffaloes and camels are source of infection for small ruminants remains unclear (Albina *et al.*, 2013). Interestingly, on one occasion a clinical case of PPRV infection was reported following experimental inoculation of calves and a further report describes an outbreak of clinical disease in buffalo caused by PPRV (Pope *et al.*, 2013). PPRV is not considered as pathogenic to cattle, domestic and wild African buffaloes (*Syncerus caffer*) although 10% of them may seroconvert when exposed to PPRV in enzootic regions (Ozkul *et al.*, 2002; Abraham *et al.*, 2005; Couacy-Hymann *et al.*, 2005). However, high case fatality rates (96%) have been reported and the disease experimentally reproduced in domestic Indian buffaloes (*Bubalus bubalis*) in India (Govindarajan *et al.*, 1997). Additionally, PPR is now recognized as an emerging disease in camelids and a respiratory syndrome was the main sign in Ethiopia and Sudan (Roger *et al.*, 2000; Khalafalla *et al.*, 2010).

The existence of sylvatic reservoirs for PPRV has been reported with infections and deaths in captive wild ungulates from several species having been described previously, such as antelopes, emsbok, bharals, ibex impala and gazelles (Abu-Elzein *et al.*, 2004; Furley *et al.*, 1987; Kinne *et al.*, 2010; Ogunsanmi *et al.*, 2003). In specific conditions, wildlife may have played an important role in PPR epidemiology in the Arabian Peninsula (Kinne *et al.*, 2010). In areas where PPRV has been present for a long time, we have some evidences that PPRV might represent a threat for wildlife. As for rinderpest, wildlife is more likely to be a victim rather than a reservoir for the PPRV (Anderson, 1995; Couacy-Hymann *et al.*, 2005).

PPR causes significant losses due to high morbidity and high mortality rates, with the latter occasionally approaching 90–100% in immunologically naive population of small ruminants, dropping to nearer 20% in endemic areas (Pope *et al.*, 2013). The rates are often higher in young animals than in adults. Whilst disease can be severe, different animals respond differently following infection in both natural and experimental settings (Diop *et al.*, 2005; Couacy-Hymann *et al.*, 2007a, b). However, there are limited data on other species such as cattle and buffaloes and particularly camel. During an outbreak of peste des petits ruminants (PPR) in camels in the Sudan, mortality rates were up to 50% (Khalafalla *et al.*, 2010).

Table 2: Selected prevalence studies of PPR in different hosts in different countries and districts of Ethiopia

Country	Species	No. tested (*)	Prevalence (%)	Reference
Africa				
Tunisia	Sheep and goat	382(cELISA)	7.45	Ayari-Fakhfakh <i>et al.</i> , 2011
Egypt	Sheep and goat	243 (cELISA)	63.4	Abd El-Rahim <i>et al.</i> , 2010
Egypt	Sheep and goat	40 (ic ELISA)	82.5	Abd El-Rahim <i>et al.</i> , 2010
Egypt	Sheep and goat	40 (FAT)	90	Abd El-Rahim <i>et al.</i> , 2010
Algeria	Sheep and goat	21(RT-PCR)	33.3	De Nardi <i>et al.</i> , 2012
Tanzania	Sheep and goat	216 (cELISA)	31	Muse <i>et al.</i> , 2012
Uganda	Sheep	106 (cELISA)	51.89	Luka <i>et al.</i> , 2011b
Uganda	Goat	210 (cELISA)	57.62	Luka <i>et al.</i> , 2011b
Kenya	Cattle	240 (cELISA)	4.2	Gitonga, 2011
Kenya	camel	160 (cELISA)	3.13	Gitonga, 2011
Nigeria	Sheep, goat cattle and Camel	2,879 (c-ELISA)	55	El-Yuguda <i>et al.</i> , 2013
Nigeria	Sheep, goat cattle and Camel	2,879 (VNT)	57	El-Yuguda <i>et al.</i> , 2013
Asia				
China	Sheep and goat	1536 (cELISA)	17.6	Wang <i>et al.</i> , 2009
India	Sheep and goat	4407 (cELISA)	33	Singh <i>et al.</i> , 2004a
India	Sheep and goat	1504 (s-ELISA)	32.8	Balamurugan <i>et al.</i> , 2012
India	Sheep and goat	4884 (cELISA)	43.46	Balamurugan <i>et al.</i> , 2011
Pakistan	Sheep and goat	1463 (cELISA)	74.9	Zahur <i>et al.</i> , 2008
Pakistan	Sheep and goat	427 (Ic ELISA)	40.98	Abubakar <i>et al.</i> , 2008
Pakistan	Sheep and goat	933 (cELISA)	51.34	Khan <i>et al.</i> , 2008
Pakistan	Sheep and goat	4548 (cELISA)	18.2	Mehmood <i>et al.</i> , 2009
Pakistan	Sheep and goat	623 (cELISA)	55.22	Abubakar <i>et al.</i> , 2011c
Kazakhstan	Sheep	542 (cELISA)	0.6	Lundervold <i>et al.</i> , 2004
Kazakhstan	Goat	137 (cELISA)	0.7	Lundervold <i>et al.</i> , 2004
Kazakhstan	Cattle	279 (cELISA)	2.2	Lundervold <i>et al.</i> , 2004

Jordan	Sheep	929 (cELISA)	29	Al-Majali <i>et al.</i> , 2008
Jordan	Goat	400 (cELISA)	49	Al-Majali <i>et al.</i> , 2008
Saudi Arabia	Sheep	992 (cELISA)	36.59	Al-Dubaib <i>et al.</i> , 2008
Saudi Arabia	Goat	962 (cELISA)	55.09	Al-Dubaib <i>et al.</i> , 2008
Europe				
Turkey	Sheep, goat and cattle	1607(cELISA)	22.4	Ozkul <i>et al.</i> , 2002
Turkey	Sheep, goat	21 (RT-PCR)	85.7	Kul <i>et al.</i> , 2007
Turkey	gazelle	82 (cELISA)	12	Gu r and Albayrak, 2010
Ethiopia				
Locality(District)				
Afar,Borena,East	Sheep	835(cELISA)	13	Abraham <i>et al.</i> , 2005
Shewa, Gambela, Jijiga	Goat	442(cELISA)	9	Abraham <i>et al.</i> 2005
	Cattle	910(cELISA)	9	Abraham <i>et al.</i> 2005
	Camel	628(cELISA)	3	Abraham <i>et al.</i> 2005
Afar (Awash Fentale)	Sheep and goat	238 (cELISA)	36.6	Delill <i>et al.</i> , 2012
Gambella(Itang)	Sheep and goat	779 (cELISA)	27.3	Megersa <i>et al.</i> , 2011
Afar (Adaar)	Sheep and goat	384 (cELISA)	38.3	Megersa <i>et al.</i> , 2011
Afar	Sheep and goat	1653 (cELISA)	15.3	Waret-Szkuta <i>et al.</i> , 2008
Amhara	Sheep and goat	5992 (cELISA)	4.6	Waret-Szkuta <i>et al.</i> , 2008
Benishangul Gumuz	Sheep and goat	729 (cELISA)	8	Waret-Szkuta <i>et al.</i> , 2008
Oromia	Sheep and goat	2290 (cELISA)	1.7	Waret-Szkuta <i>et al.</i> , 2008
SNNPR	Sheep and goat	1622 (cELISA)	1.8	Waret-Szkuta <i>et al.</i> , 2008
Somali	Sheep and goat	465 (cELISA)	21.3	Waret-Szkuta <i>et al.</i> , 2008
Tigray	Sheep and goat	900 (cELISA)	15.3	Waret-Szkuta <i>et al.</i> , 2008

* Laboratory technique employed

2.4.3. Disease pattern and seasonal occurrence

There are considerable differences in the epidemiologic pattern of the disease in different ecological systems and geographical areas (Abraham, 2005). The disease seasonality in all geographic zones is not clearly apparent. In subtropical areas, the occurrence of the disease is reported to be more common during winter and rainy seasons (Amjad *et al.*, 1996; Brindha *et al.*, 2001; Dhar *et al.*, 2002). Confinement and restricted movement of the animals, due to rainy seasons in tropical countries, may affect the nutritional status of the animals and hence predispose them to PPRV infection (Munir *et al.*, 2013). On another hand, PPR can also occur mostly during the cool, dry season in most endemic areas of Africa (Lancelot *et al.*, 2002; Abubakar *et al.*, 2009). The increase of morbidity rate is mainly observed during the years with unfavorable weather conditions and poor fodder (Kaukarbayevich, 2009). This variation is probably explained by the region-

dependent differences in animal husbandry conditions and socio-economic status of the farm owner (Munir *et al.*, 2013). The encouraging climatic factors for the survival and spread of the virus may also contribute to the seasonal occurrence of PPR outbreaks (Abubakar *et al.*, 2011b).

It has been proposed that PPRV may circulate silently, occasionally causing sporadic epidemics when the host population's immunity levels drop. The spread of PPRV is affected by both host density and birth rate and animals that survive infection are protected for life. Where particularly virulent isolates are involved and a naive population is exposed to the virus, the mortality rate is often very high. Herd animals that are in constant contact with each other, like sheep and goats, are therefore very susceptible to serious outbreaks (Anderson, 1995).

The newborn kids are susceptible to infection after 4 months of age, due to decrease in maternal protective antibodies (Srinivas and Gopal 1996; Ahmed *et al.*, 2005). Waret-Szkuta *et al.*, (2008) recently conducted a serological survey in Ethiopia and declared that age is the main risk factor for the seropositivity in small ruminants. Serological evidences revealed that antibodies occur in all age groups from 4 to 24 months indicating a constant circulation of the virus (Taylor, 1979). In Oman the disease persisted on a year round basis maintaining itself in the susceptible yearling population (Taylor *et al.*, 1990).

Therefore, an increase in incidence reflects an increase in number of susceptible young goats recruited into the flocks rather than seasonal upsurge in the virus activity, since its upsurge pend on the peak of kidding seasons. Moreover, the susceptibility of young animals aged 3 to 18 months was proved to be very high, being more severely affected than adults or unweaned animals (Taylor *et al.*, 1990).

To decrease PPR incidence and the risk of its (re-) introduction in other areas, initial steps of a PPR control program should start in highly infected regions, preferentially at the beginning of the dry season in Africa, and primarily in areas where intense animal

contacts may occur: borders, large livestock markets, pastoral areas, and so on (Albina *et al.*, 2013).

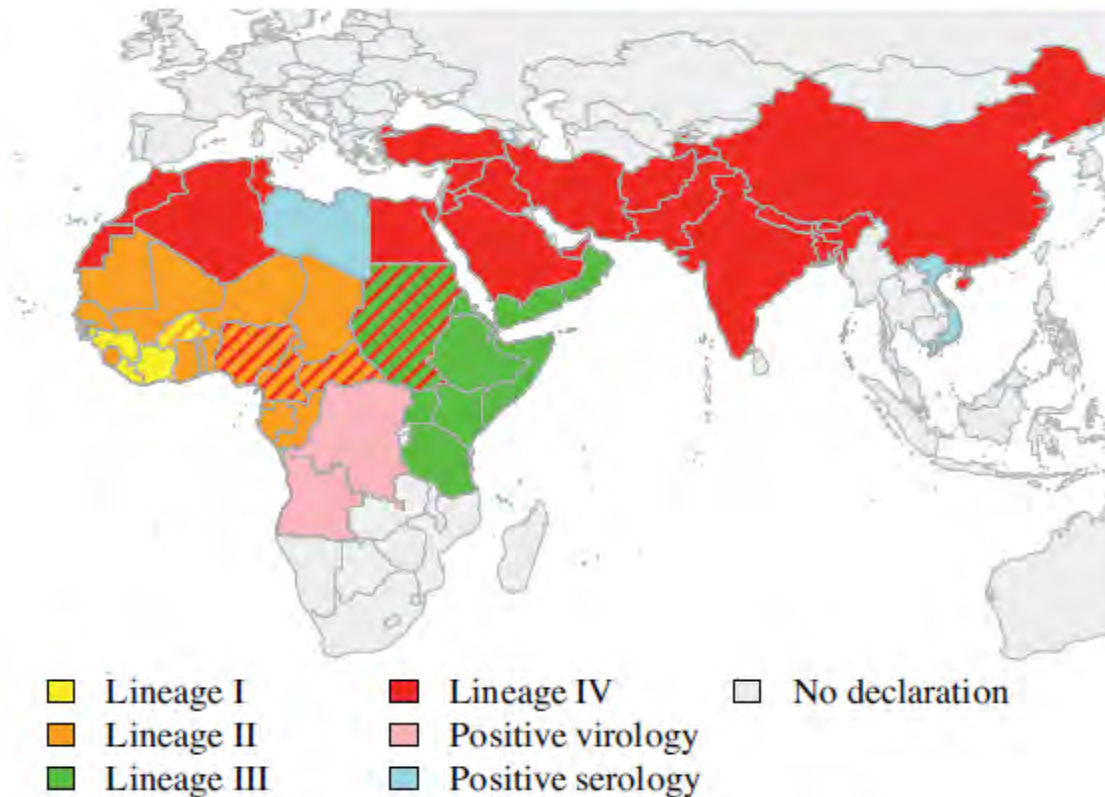
2.4.4. Molecular epidemiology

Based on the molecular characterization, strains of PPRV can be grouped into four lineages (I–IV), which are genetically distinct from each other (Kwiatek *et al.*, 2011). Classification of PPRV is being analyzed based on the sequence analysis of both F and N genes (Shaila *et al.*, 1996; Couacy-Hymann *et al.*, 2002; Kerur *et al.*, 2008); however, parallel comparison of PPRV strains has proposed that N gene is most divergent and hence most appropriate for molecular characterization of closely related isolates (Kwiatek *et al.*, 2007). Both are well conserved genes with $\approx 10\%$ nt mean variability between the most distantly related sequences; this variability can exceed 30% on some parts of the sequence (Kwiatek *et al.*, 2011). The virus has been recognized to occur as only one serotype among four lineages (Shaila *et al.*, 1996). This classification of PPRV into lineages has broadened our understanding of the molecular epidemiology and worldwide movement of PPR viruses (Kerur *et al.*, 2008).

The three first lineages were historically settled in Africa according to the apparent spread of the virus from West to East Africa (Banyard *et al.*, 2010). Lineage III is also common to south part of Middle East (Yemen, Qatar and Oman), reflecting the major movements of sheep and goats from the Horn of Africa to these countries (Baron, *et al.*, 2011) and unexpectedly once southern India. The fourth lineage was until recently confined to Asia, including Turkey and the Arabic peninsula. Within a remarkably short time, it spread to a large part of the African continent. In 2008, an extensive epidemic of the PPRV lineage IV occurred in Morocco. This finding was particularly relevant as it marked further geographical spread of this lineage outside eastern and western Africa and its first detection in northern Africa. It is now found from Sudan to northern Africa (Algeria), as well as central Africa and the Gulf of Guinea (Khalafalla *et al.*, 2010;

Kwiatek *et al.*, 2011; Fig. 2). Interestingly, older outbreaks of PPRV in Sudan were lineage III, while recent outbreaks have been lineage IV, suggesting a change in animal movement patterns in that region (Baron, *et al.*, 2011). Furthermore, because of these outbreaks in Morocco and the existing commercial trade between Morocco and both Algeria and Spain, the situation raised huge concern owing to the increased risk of introduction of the disease into free zones in northern Africa and into Europe (FAO, 2009; Khalafalla *et al.*, 2010). In Senegal and Mauritania, a similar scenario has occurred with lineage II, originating from Central Africa and moving to West Africa whereas in the 1980s, lineage I was the dominant, if not the single lineage found there. The presence of the two African lineages in Asia alongside a distinct Asian lineage gave an indication of the trade route of spread of the disease (Abraham *et al.*, 2005).

It is still unclear whether differences between lineages are merely reflecting geographical speciation or if they are also correlated to pathogenicity variability between isolates (Banyard *et al.*, 2010). The proper understanding of lineage distribution in a specified region is essential when choosing the appropriate homologous prototype to ensure efficient immunization. The continued application of heterologous vaccine candidates hitherto not prevalent may lead to generation of novel lineages, or allow the existing population to evade protection, especially in RNA viruses. Therefore, identification of the lineage is a pre-requisite for fruitful diagnosis, epidemiology and control (Munir *et al.*, 2012b).



Source, Albina *et al.*, (2013)

Fig. 3: Worldwide cumulative distribution of the four PPR virus lineages. Different colors show different lineages and hatched bars represent the last identified lineage in the corresponding country. The pink color indicates virological evidence of PPR infection. The blue color indicates serological evidence of PPR infection but no virus isolated. The grey color indicates missing information or disease never reported.

2.4.5. Drivers (risk factors) for PPR transmission (challenging its eradication)

PPRV is mainly transmitted by the aerosol route and as such often requires close contact between animals to spread within a herd (Banyard *et al.*, 2010; Abubakar *et al.*, 2012). The affected animals during the febrile stage of disease are important source of transmission (Braide, 1981). Although no carrier state for PPRV has been defined, virus may be shed during subclinical cases or during incubation periods (Abubakar *et al.*, 2012). Couacy-Hymann *et al.*, (2007a) confirmed in an experimental infection that infected animals could transmit PPRV before the onset of clinical signs. The year after,

Ezeibe *et al.*, (2008) studied the shedding of virus during the post-recovery state of the animal, and realized that goats infected with PPRV can shed virus antigens in feces for 11 weeks after complete recovery (Ezeibe *et al.*, 2008).

Animals affected by PPR shed the virus in exhaled air, in secretions and excretions (from the mouth, eye and nose, and in feces, semen, and urine) approximately 10 days after the onset of fever. Sneezing and coughing by the infected animal can spread infection, while the transmission between animals in the vicinity can occur through inhalation (over a distance of 10 m) or, unlikely, through inanimate objects (fomite) due to its rapid inactivation in external dry conditions. Spread through ingestion and conjunctival penetration, and by licking of bedding, feed, and water troughs, is also not uncommon. Infection may spread to offspring by feeding them the milk of an infected dam (Munir *et al.*, 2013).

The presence of mixed populations (i.e. flock of sheep and goats) and the introduction of new animals into a flock/herd or the return of animals that were not sold in the market to the village is major risk factors for the PPR spread (CFSPH, 2008). In pastoral areas, livestock trade, nomadic herding (FAO, 2009) and the congregation of susceptible populations close to watering points during dry seasons and/or in livestock markets play an important role in spreading the disease.

Stress related to changes in the farming procedures (change of diet, habitat, and development of intensive fattening units) also affords increased opportunity for PPR transmission (Abraham *et al.*, 2005; EMPRES WATCH, 2008). Furthermore, limited fodder availability often, leads to nutritional deficiency, resulting in increased susceptibility to infection. Consequently, large numbers of animals become infected during this period and these animals then help to maintain the circulation of the virus throughout the year by frequent animal-to-animal transmission (Abubakar *et al.*, 2011b). These factors may play a key role in the transmission of PPR infection.

The other challenge is the role of wildlife in the PPR spread. This is suggested by Kinne *et al.*, (2010) who isolated the virus from different wild small ruminants kept under semi-free-range conditions in the United Arab Emirates (UAE). Sequence analysis of the N gene indicated that the virus belongs to lineage IV, and was different from the viruses already isolated from the Arabian Peninsula (Kinne *et al.*, 2010). Further analysis indicated that these isolates are more closely related to Chinese ones rather to the expected Saudi Arabian isolates. The origin of this new PPRV strain in the region has not been investigated, but it highlights the role of wild ruminants as a possible threat to domestic small ruminants.

2.5. Recent diagnostic methods

Earlier studies have suggested that PPR might have been around for quite some time in different countries but was wrongly diagnosed (EMPRES, 2008). PPRV is routinely identified on the basis of clinical examination, gross pathology, histological findings, and laboratory confirmation (Abubakar *et al.*, 2011a). Clinical signs and lesions can be misleading for PPR diagnosis since other diseases, including pasteurellosis or contagious caprine pleuropneumonia, have similar consequences. It has also been confused with rinderpest because of the clinical similarity (Haas and Barrett, 1996).

Laboratory techniques that were used in diagnosis were virus neutralization test (VNT), agar gel immunodiffusion test (AGID), counter immuno-electrophoresis (CIEP) and virus isolation which has been time consuming and laborious (Balamurugan *et al.*, 2007). Effective implementation of control measures for PPR require rapid, specific and sensitive methods for diagnosis (Munir *et al.*, 2012a). 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 (Ularamu *et al.*, 2012).

Fortunately, a number of serological and molecular diagnostic tests are currently available for the detection of PPRV or confirmation. These laboratory tests can be divided into those that look for the virus (PCR or immunocapture ELISA [i_c-ELISA]), used to detect acute infection, and those that look for antibodies against the virus (competitive ELISA [cELISA]), used for serum surveillance studies and to estimate how widespread infection has been in a flock or area (Baron, *et al.*, 2011).

Currently, the OIE recommend the use of the competitive PPRV specific anti-H monoclonal based ELISA (cH-ELISA) and virus neutralization tests for detection of antibodies to PPRV (OIE, 2013). The most reliable and rapid test for antibody detection is a competition ELISA based on a monoclonal antibody directed against the virus nucleoprotein. Because VNT necessitates cell and virus cultures, and thus takes several days before result outcome, it is not used as a routine test. It is mostly used in reference laboratories to confirm unclear results (Albina *et al.*, 2013). However, several alternatives exist including the indirect N ELISA (Ismail *et al.*, 1995), immunofiltration (Raj *et al.*, 2008), a novel sandwich ELISA (Saravanan *et al.*, 2008), haemagglutination (HA) and haemagglutination inhibition (HI) tests (Raj *et al.*, 2008; Saravanan *et al.*, 2006; Manoharan, 2005; Raj *et al.*, 2000; Ezeibe *et al.*, 2008) and latex agglutination tests (Keerti *et al.*, 2009).

Detection of PPRV antigens can be performed using a variety of tools including immunocapture ELISA, counter immunoelectrophoresis (CIEP) or agar gel immunodiffusion (AGID). CIEP and I_c-ELISA can distinguish PPRV from RPV, but the AGID test cannot differentiate these two viruses. AGID is also relatively insensitive, and may not be able to detect small quantities of viral antigens in milder forms of PPRV (Banyard *et al.*, 2010).

However, HA and HI tests, being simple, cheaper and comparatively sensitive, can be used for routine screening purposes in control programmes (Munir *et al.*, 2009; Osman *et al.*, 2008).

The first i_c -ELISA was used by Saliki (1994) for identifying PPRV, and used a polyclonal capture antibody. A subsequent version using monoclonal antibodies for both capture and detection was developed by Libeau and coworkers (Libeau and Lefevre 1990, Libeau *et al.*, 1994). This test has the advantages of simplicity (low technology requirements) and rapidity, shows good sensitivity and works on samples that have been stored at room temperature (ie, when the virus infectivity would have been destroyed), and is now considered a definitive diagnostic test by the World Organisation for Animal Health (OIE, 2013).

An extension of the principles of the i_c -ELISA can be found in the use of ‘pregnancy test’-type pen-side tests based on the chromatographic strip test technology and the dot ELISA that can be performed without the need for equipment or technical expertise (Hussain *et al.*, 2003; Aslam *et al.*, 2009). Such a test was developed for RPV diagnosis towards the end of the eradication campaign (Bruning *et al.*, 1999, Wambura *et al.*, 2000); a similar test for PPRV is currently under development at the Institute for Animal Health (Bruning-Richardson *et al.*, 2011).

Virus isolation in cell culture can also be attempted with several different cell lines, although recovery of virus is not always successful. Previously, a marmoset-derived cell line (B95a) was primarily used (Sreenivasa *et al.*, 2006) although primary lamb kidney or African green monkey kidney (Vero) cell cultures have also been successful (Mahapatra *et al.*, 2006). African green monkey kidney cells (Vero) have been for a long time the cells of choice for the isolation and propagation of PPRV. However, some isolates may not grow well in these cells. Recently, transformed monkey cells expressing sheep/goat signaling lymphocytic activation molecules (SLAM or CD150), the virus cellular

receptors, have been shown to possess increased sensitivity (Adombi *et al.*, 2011). Generally, cultures are examined for cytopathic effect in the days following infection of a monolayer with suspect material; the identity of the virus can be confirmed by virus neutralization or molecular techniques (Singh *et al.*, 2009). Techniques for virus isolation cannot be used as routine diagnostic tests as they are time-consuming and cumbersome (OIE, 2013). Moreover, the preservation of samples collected under field conditions is not always adequate for successful laboratory results. Virus isolation does, however, play an important role from a research perspective.

The most popular techniques for virus detection are based on molecular biology. For molecular detection, standard RT-PCR (Couacy- Hymann *et al.*, 2002) has now been superseded by the most sensitive techniques, real-time RT-PCR assays specific for PPRV (Bao *et al.*, 2008; Kwiatek *et al.*, 2010; Batten *et al.*, 2011) and loop-mediated isothermal amplification techniques (Wei *et al.*, 2009). This assay utilises RNA extracted from field samples using robotic extraction methods allowing high throughput, specific diagnosis of PPRV, whilst reducing cross contamination of samples and potential for human error (Batten *et al.*, 2011).

The generation of a standard RT-PCR product is, however, necessary in order to perform sequence analysis and subsequent phylogenetic characterization of novel virus isolates (Banyard *et al.*, 2010). The sequence of the PCR products has proved very useful in tracking the movement of the virus. There are no serotypes of PPRV but, as mentioned, there are clear lineages based on phylogenetic analysis of virus sequence. The utility of lineage identification lies in the information it provides on the likely origin of the virus causing a new outbreak. Diagnosis of acute samples in reference laboratories is now based almost entirely on either gel-based or real-time PCR (Baron, *et al.*, 2011).

2.6. Progress and obstacles in vaccine development

The most effective way to control PPR is mass immunization of small ruminants as often, farmers in areas where the virus is endemic are unable to afford and implement the strict sanitary control measures, including the stamping out policy, required to contain the virus. Therefore, the controls of PPR require an effective vaccine and for this purpose several vaccines including both homologous and recombinant vaccines have been developed (Abubakar *et al.*, 2011a).

The first homologous, attenuated live cell culture vaccine was developed by attenuating the PPRV Nigeria 75/1 isolate by Diallo *et al.*, (1989), which belongs to lineage 1 and has been used extensively in Africa and the Middle East to try to suppress outbreaks; the vaccine gives protection for at least three years (OIE, 2013). However, it has a low thermal stability: half-life of 2–6 h at 37°C after reconstitution (Diallo, 2004). To overcome this, the vaccine strain has been mixed with cryo-protectant mixture containing trehalose. Thus, preservation of sufficient virus titer could be extended to 5–14 days at 45°C in the lyophilized form, and 21 h at 37°C after reconstitution (Worrall *et al.*, 2000; Silva *et al.*, 2011). These thermostabilizing additives are compatible with the shipment of the vaccine to remote areas without the need for a cold chain. Two thermostable vaccines were developed and their thermo-stability when checked at both 37°C and 40°C, demonstrated a shelf life of 7.62 and 3.68 days, respectively (Sen *et al.*, 2010). Heavy water has been reported to enhance the thermo-stability of PPRV vaccines.

At least three similar PPRV vaccine candidates have been developed in India and licensed for use: Sungri 96, Arasur 87 and Coimbatore 97 (Saravanan *et al.*, 2010). This vaccine, however, also has certain limitations, including requirement for cold-chain maintenance, and inability to differentiate infected from vaccinated animals (DIVA).

As with all members of the Paramyxoviridae family, PPRV is heat sensitive; this is a serious drawback for the efficient use of the live attenuated vaccine in the endemic areas, which have hot climatic environments. In addition these regions usually have poor infrastructures, being difficult to sustain a cold chain to ensure the maintenance of vaccine potency. Lyophilization in the presence of suitable excipients is a prevailing approach to stabilize such type of biological products (Silva *et al.*, 2011).

Alternative thermotolerant PPR-recombinant poxvirus vaccines have been engineered in the past (Jones *et al.*, 1993; Diallo *et al.*, 2002, 2007; Berhe *et al.*, 2003; Chen *et al.*, 2010), which should provide simultaneous protection against both diseases, although none of them have yet been launched in the market and used in the field. Any of these vaccines would have the additional advantage over the attenuated PPRV vaccine of being less heat labile, although the experience gained during the rinderpest eradication campaign showed that good freeze-drying techniques could provide highly stable vaccine preparations, essentially eliminating the need for a cold chain for vaccine delivery (House and Mariner, 1996).

To improve the control of PPR in the field, it might be interesting to develop a DIVA vaccine (differentiating infected from vaccinated animals) that could provide a practical and useful means to control the disease, especially in tropical countries. Such a DIVA vaccine associated to a performing DIVA diagnosis may only be important when PPR becomes close to elimination, to reveal a low-noise PPRV transmission and trigger the reinforcement of vaccination campaigns and disease surveillance based for instance on a test-and removal strategy (elimination of animals tested with infection antibodies) to speed up the eradication (Albina *et al.*, 2013). It could also be a worthwhile tool in case of re-emerging outbreaks in a free-zone since an emergency vaccination plan and a parallel test-and-removal control strategy could rapidly blow out a new epizootics. However, the DIVA vaccine is not an urgent issue because the existing vaccines, possibly improved for better thermotolerance, are fully efficient for the early stages of PPR

control. Recombinant poxviruses are theoretically DIVA vaccines since the antigens not included in the vaccines can serve in the serological tests (Albina *et al.*, 2013).

Another interesting option is to modify the existing and widely used attenuated vaccine. Several research groups are working on this possibility by using reverse genetics (Minet *et al.*, 2009; Hu *et al.*, 2012). Again, the time before a commercial vaccine will become available may cover several years. Several African and Middle East laboratories have the capacity to produce and deliver tens of millions of PPR vaccine doses within months, like it was the case in Morocco after PPR emergence in 2008. A major point is the necessity of reliable quality controls before vaccine batch release. With this respect, the PANVAC laboratory based in Debre Zeit is an outstanding tool to ensure the quality of PPR vaccines used in the vaccination campaigns. The number of vaccine doses per vial will also need to be adapted to field situations. For instance, vials of >100 doses should be avoided in areas where sheep and goat flocks are tiny. Given adequate manufacturing process, most of the eradication program can be achieved using the current vaccines (Albina *et al.*, 2013).

2.7. Research needs

Key research areas that include rapid pen-side diagnostic tests and a 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. Furthermore, development and technology transfer of an efficacious thermo-stable vaccine against PPR (and sheep and goat pox) in the short term remains an area for further research (FAO, 2013).

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 and camel in the transmission of PPRV. Rinderpest was regularly observed in many different species of wildlife, including buffalo, giraffe and several species of antelope; however, vaccination of livestock herds against rinderpest led to the disappearance of the disease from wildlife populations, showing that the wildlife were not acting as reservoirs of infection, but were being infected by the virus circulating in the domestic livestock. It is essential to fully understand the role of wildlife in the spread and potential maintenance of PPRV in the environment in order to be able to initiate successful control strategies (Baron *et al.*, 2011).

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. 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).

Another major gap for the success of PPR control is the lack of economical assessment of control strategies, and even of PPR cost. Such information would be useful to help veterinary services in convincing governments and international organizations to support and fund PPR control (Albina *et al.*, 2013).

3. MATERIALS AND METHODS

3.1. Study area

The study area was purposively targeted the rift valley escarpments of Eastern Amhara region which encompasses the lowlands of North Shoa zone, Oromia zone in Amhara region, South Wollo and North Wollo zones of the region. It includes districts immediately adjacent to the pastoral areas in Afar region of the country and epidemiologically closely linked to these areas through seasonal admixture of the herds during grazing and marketing. The study was carried out in 18 villages selected from five districts: Kobo and Habru districts from North Wollo, Werebabo district from South Wollo, Bati district from Oromia special zone and Kewet district from North Shoa (Fig. 4 and Table 3).

Kewet District

Kewet District is about 225 km to the north of Addis Ababa along Dessie road. It is located at 11° 55' N latitude and 37° 20' E longitude at an altitude of 1380 m.a.s.l. The area has an average annual rainfall of 1007 mm, with short rain between March and April and long rain between June and September and annual mean minimum and maximum temperature of 16.5 and 31°C, respectively (BoA, 2000).

Bati District

Bati district is one of the food insecure districts in the Amhara National Regional State and is situated 420 km North East from the capital city of the country, Addis Ababa. The geographical location of the district is 10⁰55' and 11⁰30' N latitudes and 39⁰50' and 40⁰15'E longitude (BoA, 2007). It has an altitude range of 1001-2500 m.a.s.l. The district

shares boundaries with Afar National Regional State in the east, Kalu district (South Wollo Zone) in west, Werebabu (South Wollo Zone) and Dawa Chefa district (Oroimiya Zone) in the south. The study area is agro ecologically classified as mid altitude (*Weyna dega*) 19% and lowland (*Kola*) 81%. Rainfall is bi-modal and the short rainy season (*Belge*) starts in January and extends to April. The long rainy season (*Meher*) starts in June and extends to September. The rainfall distribution in the study area is erratic in nature and from 500 to 1000 mm annually while the temperature ranges from 18 to 36°C annually (BoA, 2007).

The district is characterized by mixed farming system, where the rural people of the district are dependent on both crop and livestock production for their living. According to Bati district land use data (2007), the district has a total of 124,696 ha; of this total 8.96% is cultivated, 15.16% is grazing land, 0.5% is forest, 49.38% is bush land, 6.8% is used for settlement, and over 19% is marginal land or waste land. The landscape of this district classified as rugged terrain (42%), mountainous (20%), gorge (28%) and plane (10%). Type of soil found are black, red, sandy and gray soils and make up are about 23%, 12%, 11% and 54%, respectively (BoA, 2007).

Habru District

Habru is one of the districts in North Wollo zone. It is bordered on the south by the Mille River which separates it from the South Wollo Zone, on the west by Guba Lafto, on the north by the Logiya River which separates it from Kobo, and on the east by the Afar Region. The main Town of the District Mersa is located at 491 km North of Addis Ababa. The altitude of this district ranges from 700 meters above sea level where the Mille enters the Afar Region, to 1900 meters at its westernmost point. The study area is agro ecologically classified as highland (*Dega*) 1%, mid altitude (*Weyna dega*) 24% and lowland (*Kola*) 75% (BoA, 2013).

Werebabo District

Werebabo is one of the districts in the Amhara Region of Ethiopia. Part of the South Wollo Zone, Werebabu is bordered on the south by Kalu, on the west by Tehuledere, on the north by the Mille River which separates it from the North Wollo Zone, on the east by the Afar Region, and on the southeast by the Mio River which separates it from the Oromia Zone.

The western part of Werebabu lies in the Ethiopian highlands, with the eastern part stretching down to the lowlands of the Afar Region; elevations range from 700 meters above sea level where the Mille leaves the district to 2700 meters at its southern most point. The study area is agro ecologically classified as highland (*Dega*) 4%, mid altitude (*Weyna dega*) 40% and lowland (*Kola*) 56% (BoA, 2013).

Kobo District

Kobo district is found along the Northern boundary of the Amhara National Regional State in Northern Ethiopia. In terms of geographic coordinates, the study area is located within the Gobu watershed described by 12°10' and 12° 13' North Latitude and 39° 22' and 39° 33' East Longitude. The highest long term mean monthly rainfall is 197 mm in August while the lowest mean monthly rainfall is 10 mm in December. Mean maximum temperature of 34°C and mean minimum of 12°C for June and December respectively based on records of the nearby meteorological stations at Kobo Station. The rainfall pattern is bimodal, i.e. the short rains (Belg-April and May) and long rains (Kiremt-July and August). The hottest and coldest months of the study area are May-June and November-December respectively (BoA, 2013).

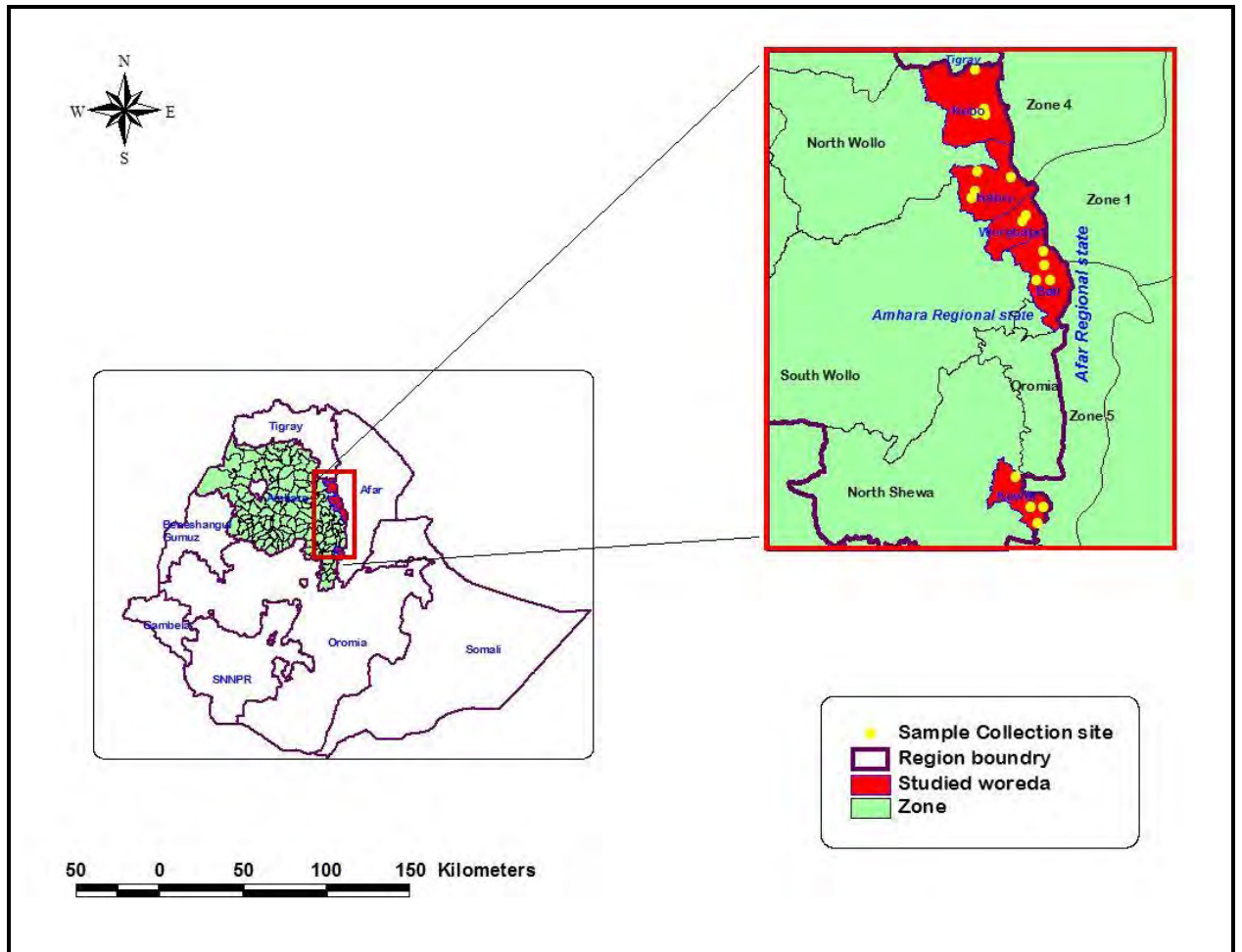


Fig. 4: Map of Ethiopia showing Amhara region, study districts and sampling areas.

3.2. Study population, sampling scheme and sample size

The study population was small ruminants that are apparently healthy as well as those showing clinical signs that resembles of PPR signs.

Multistage sampling (Dohoo *et al.*, 2003), with four hierarchical stages, was used as sampling strategy. The zones and districts bordering Afar regional state were sampled purposively to address the perceived risk due to either contact with trade or nomadic animals through shared grazing and watering points. The first level of selection was the

zones, within each of the selected zones, specific districts were also selected. Peasant Association (PAs)/villages and individual animal were selected by random sampling. Herds within the same village that share grazing areas and watering points were considered as single-level clusters and sampling units, with very little within-flock variation. A village was, therefore, considered to constitute a flock.

The sample size was computed as suggested by Waret-Szkuta *et al.*, (2008) and Dohoo *et al.* (2003): The sample size required for the seroprevalence study was determined according to Thrusfield (2005).

$$n = z^2 \times [P_{\text{exp}} (1-P_{\text{exp}}) / d^2],$$

Where: $z = 1.96$, $P_{\text{exp}} = 0.5$ and $d = 0.05$ (the desired level of precision or accuracy). The required sample size was accordingly calculated as $n = 384$.

However, PPR is a highly contagious disease. To obtain a similar accuracy to that with simple random sampling, the sample size had to be recalculated. The aim was to sample at least 54 animals from each village, and based on previous study on analysis of national serological data by Waret-Szkuta *et al.*, 2008, the median rate of PPR homogeneity rho (p) was estimated to be 0.029. Therefore, the sample size was subsequently recalculated as:

$$N = n \times [1 + p \times (m - 1)],$$

Where $n = 384$, $p = 0.029$ and $m = 54$, representing the average number of animals to be sampled from each village (Dohoo *et al.* 2003). Therefore, the new sample size was equal to 974.208 animals which accommodate the lack of independence between small ruminants belonging to a given kebele (Waret-Szkuta *et al.*, 2008) and 18 villages were included in this study. The design effect (D) was calculated as 2.537 according to:

$$D = 1 + (m - 1) \times p$$

The standard error (S) was calculated as 0.013 according to:

$$S = \sqrt{p \times (1-p) \times D/n}$$

The sampling frame from which the 18 study villages were randomly selected was obtained from respective district agriculture office.

Table 3: Study areas, zonal sheep and goat population and respective number of sample

Zone	Zonal Population		Districts selected	No. of sample	PA and Village selected
	Sheep	Goat			
North Wello	862,546	501,259	Kobo	222	Addis Kegn, Addis Alem, Dibi, Sodu
North Wello	862,546	862,546	Habru	210	Tis Keye, Jarota, Mehal Amba, Dire Roka
South Wello	1,948,943	720,700	Werebabo	98	China, Arebat
Oromia	86780	164,891	Bati	223	Chekorti, Felana, Chachatu, Salemene
North Shewa	1,644,881	732,433	Kewet	216	Kure Beret, Sefi Beret, Medina, Yelen
Total sample size				969	

3.3. Study design and data collection

A cross-sectional study design was employed between November 2013 and April 2014 to collect epidemiological data and samples.

3.3.1. Retrospective analysis of epidemiological data on PPR outbreak

Five years retrospective data on PPR outbreak was used in the present study. The source of the data on the outbreaks and epidemiology of PPR infection in various regions of the country was the Federal Ministry of Agriculture, Epidemiology unit, Ethiopia. The data were transmitted from the local field veterinarians to the ministry through the officers of the districts concerned.

Retrospective description of epidemiological data on number of PPR outbreaks, sick and deaths, animals at risk and species involved were conducted for the period January 2009 to December 2013. Monthly reports at district level were obtained from the region.

3.3.2. Active field investigation and Questioner survey

A semi-structured questionnaire was developed and pre- tested. The questionnaire was administered to those small ruminant owners showed their willingness to participate in the study in the selected villages. Each interview took about 20–30 minutes. From each districts about 12 to 15 people in each PAs were selected for the survey. In each village, flock owners were interviewed to reveal information regarding flock size, age and sex, health statuses, grazing management, raising type, introduction of new animals, access to veterinary services, clinical signs of disease encountered, number of diseased and dead animals.

Age was approximated and classified as young (6 months to ≤ 1.5 year) and adults (> 1.5 years). Health status data was collected by recording occurrence of enteritis-stomatitis syndrome and respiratory distress, overall number of sick animals (used to compute morbidity) as well as overall and specific deaths associated with observed clinical cases (used to compute crude and case fatality of PPR). Close-ended questions were coded and entered in a excel spread sheet. Open-ended questions were collected and similar answers were grouped.

In addition, an observational study on clinical cases was conducted during the occurrence of an outbreak and photographed using digital camera. Samples for viral culture, isolation and identification were collected from animals that exhibited clinical signs suspected to be PPR.

3.3.3. Serological study

A total of 969 serum samples were collected from sheep and goats in the study districts. The aim was to determine the level of antibody in the serum/herd immunity in vaccinated areas as well as the sero-prevalence in high risk areas of infection in non-vaccinated areas. Initially, blood samples were collected by jugular-vein puncture with plain vacutainer tube from each animal gathered at the immediate veterinary clinic of respective PA/Kebele. Supplementary information on potential risk factors (such as animals age and sex, health statues, grazing management, raising type, introduction of new animals, access to veterinary services) was recorded during blood sampling. The samples were labeled accordingly to allow identification of each animal, flock sampled and studied village and kept in a slanted position overnight to allow serum separation via clotting blood samples. Serum was decanted and aliquoted into 2 mL cryovials before being transported and temporally stored in cold boxes. Finally, the serum samples were transported in an ice box chilled on ice packs to the National Animal Health Diagnostic and Investigation Center Laboratory, Sebeta, where serological analysis was carried out using cELISA. Upon arrival, the serum was centrifuged to remove the remaining red blood cells before being transferred to new cryovials and stored at -20°C until processed.

A monoclonal antibody (MAb) based competitive Enzyme Linked Immunosorbent Assay (cELISA) (Diallo *et al.* 1995; OIE, 2013) was used for the detection of antibodies directed against the nucleoprotein of the PPR virus using approved competitive ELISA kit as described by Libeau *et al.* (1995).

Briefly, the ELISA wells were coated with purified recombinant PPR nucleoprotein (NP); the samples to be tested and the controls were added to the microwells. Anti-NP antibodies, if present, form an antibody-antigen complex which masks the NP epitopes. An anti-NP-peroxidase (HRP) conjugate was added to the microwells and incubated. It fixes to the remaining free NP epitopes, forming an antigen-conjugate-HRP complex. After washing (to eliminate the excess conjugate), the substrate solution (TMB) was

added and the resulting coloration depends on the quantity of specific antibodies present in the sample. Stop solution (sulfuric acid) was added to each well in order to stop the reaction. The microplates were read with ELx800 Absorbance Microplate Reader (Biotek® Instruments, Inc. USA) with an inference filter of 450 nm and connected to a computer loaded with Gen 5™ software for automated reading and calculation of the competition percentage (S/N %) values.

The OD (optical density) values of each sample were converted to S/N % by using the following formula:

$$\text{S/N \%} = [\text{OD}_{\text{sample}} / \text{OD}_{\text{NC}}] \times 100$$

The samples with S/N less than or equal 50 % were considered as positive.

The same procedure was used in this study to convert the OD values to percentage inhibition for PPR detection by using the following formula:

$$\text{PI} = [100 - (\text{OD}_{\text{sample}} / \text{OD}_{\text{NC}})] \times 100$$

An inhibition of more than 50 percent was considered positive.

3.3.4. Antigenic detection using Immuno-capture ELISA (I_C-ELISA)

A total of 32 samples, comprising 18 swab samples (nasal, oral, and ocular and gum debris) and 14 heparinized whole blood samples were collected from the suspected animals for the presence of PPR viral antigen. The swab samples were collected using sterile swabs which were placed in a viral transport media (VTM) containing PBS, antibiotic and antifungals. I_C-ELISA was also used to detect PPR viral antigen in harvested inoculated CHS-20 cells.

Immuno-capture ELISA for the detection of PPR virus was carried out at the National Animal Health Diagnostic and Investigation Center Laboratory, Sebeta, using ID Screen® Sandwich ELISA kit as described by Libeau et al. (1994). The kit was imported from FAO/OIE Reference Laboratory for PPR, CIRAD, Montpellier, France. It used an

anti-nucleoprotein (N) capture antibody and an anti-N monoclonal HRP antibody to demonstrate the presence or absence of PPR viral particles in the specimen. It was applied according to the manufacturer's instructions (ID.Vet). Samples to be tested and controls were added to the microwells. If PPRV present, forms an antibody-antigen complex. After washing, an anti-PPRV-N Mab-HRP conjugate was added, forming an antibody-antigen-Mab-HRP complex. After washing (to eliminate the excess conjugate), the substrate solution (TMB) was added. The resulting coloration depends on the quantity of PPRV present in the sample to be tested. In the presence of PPRV, a blue coloration appears which becomes yellow after addition of the stop solution (sulfuric acid). In the absence of PPRV, no coloration appears. The microplates were read with ELx800 Absorbance Microplate Reader (Biotek® Instruments, Inc. USA) at 450nm. The reader was connected to computer loaded with Gen 5™ software, which was used to automate the reading and calculation of percent positivity (S/P %) values. The OD (optical density) values of each sample were converted to percentage positivity by using the following formula:

$$S/P \% = [OD_{\text{sample}} - OD_{\text{NC}} / OD_{\text{PC}} - OD_{\text{NC}}] \times 100$$

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

3.3.5. Molecular detection of the virus nucleic acid

RT-PCR for the amplification of part of the N gene

28 samples including the previous 18 swab samples, additional 10 nasal swab samples and those inoculated to CHS-20 cells and harvested were examined for the presence of PPRV RNA by one step reverse transcription- polymerase chain reaction (RT-PCR) assays (OIE, 2013).

Primarily, the RNA extraction from samples was done using commercial RNA extraction kit (Qiagen® RNeasy Mini Kit, courtaboef, France) as per the manufacturer's

instructions. Reverse Transcription- Polymerase Chain Reaction (RT-PCR) was performed for the N-gene of PPRV using 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 Couacy- Hymann *et al.* (2002).

The master mix contained the following reagents: 7.5 µl of RNase-free water, 5 µl of 5X PCR buffer, 1 µl of dNTPS mix (10mM each), 1.5 µl 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 22.5 µl of the prepared master mix and 2.5 µl 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 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 5 min in an Applied Biosystem 2700/2720 Thermal cycles PCR machine.

10 µl of each PCR product (amplicon) were analysed by gel electrophoresis at 120v/80mA for 60 min on 1.5% of agarose gel in Tris-borate-ETDA buffer. The gel was stained with ethidium bromide and the DNA bands were visualized by UV transilluminator and the image was transferred to computer.

3.3.6. Cell culture and virus isolation

Anti-mortem samples (oral, ocular, nasal swabs and gum debris) were collected from active cases in a sterile universal tube containing virus transport medium (VTM) with the

aim of virus isolation. Samples were kept on icebox containing ice pack during collection procedures and until shipment to the laboratory. 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 supernatant were collected and samples taken from one outbreak area or district were pooled together assuming that the same virus could cause the outbreak in the population. Accordingly, 4 pooled samples were processed in cell culture from 4 respective different districts for virus isolation.

The cell culture inoculation was carried out according to the method of Adombi *et al.*, (2011) in a cell line of CHS-20 (Appendex V). Monolayer cell cultures were inoculated with the suspect pooled samples (swab material) and examined daily for evidence of cytopathic effect (CPE). Positive cultures were retested with Ic- ELISA, RT-PCR and IFAT.

3.4. Data management and analysis

Districts, PAs, villages, vaccination status of the village and individual animal data were stored in Microsoft Excel 2007. Descriptive statistics of the explanatory variables examined in the study for the animal and flock level were computed using Epi Info software. The prevalence was determined by dividing the total number of positive samples by the total number of samples (Dohoo *et al.* 2003). Proportions were calculated for seroprevalence *visa-vis* fixed factors that included animal species, sex and age, districts and village. Univariable analysis for the proportions was carried out using Chi-square analysis in Epi Info software version 3.5.1 (Centre for Disease Control and Prevention) to assess association with the flock size, age and sex, health statuses, grazing management, raising type, introduction of new animals, access to veterinary services. A confidence limit of less than 5% was used to indicate a significant level. All variables with $P < 0.05$ (two-sided) in the univariable analysis were further tested by multivariable logistic regression model to assess their effect on PPR seropositivity. Study village maps

were created using ArcGIS version 3.2. Choropleth maps were also produced to show the distributions of outbreak report by *wereda*.

4. RESULTS

4.1. Temporal and spatial distribution of PPR using 5 years retrospective data

4.1.1. Temporal distribution of PPR

Between 2009 and 2013, a total of 832 PPR outbreaks were reported from different regions and districts of the country. The highest number of outbreaks despite the lowest case fatality rate was reported in 2010. The monthly overall outbreak pattern of the disease is shown in Fig. 5. The incidence of PPR outbreaks increased gradually during September and October and with a peak in November. The lowest number of outbreaks was seen in the month of June. The study showed clearly that the greatest number of outbreaks was observed after the main rainy season.

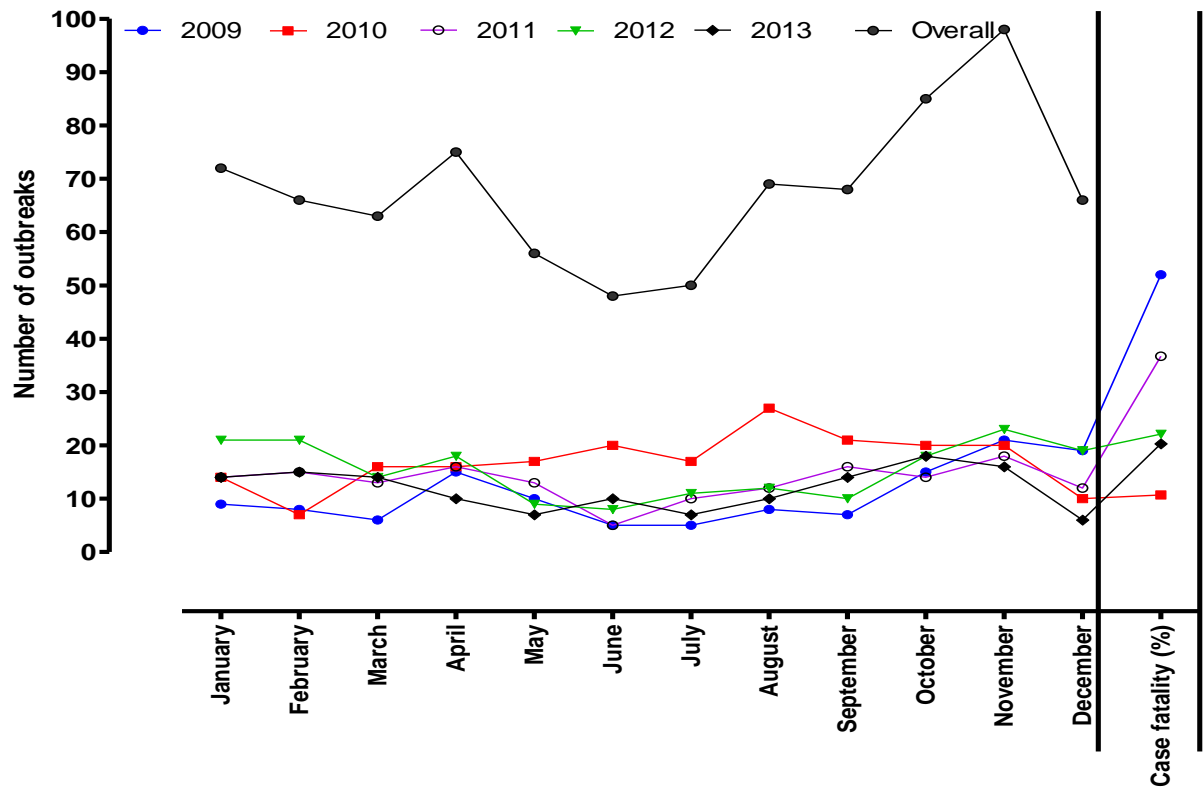


Fig. 5: PPR seasonal disease pattern and case fatality rate during 2009-2013

4.1.2. Spatial distribution of PPR

Region wise, Oromia reported the highest number of outbreak followed by Amhara and the least was recorded in Diredawa followed by Gambela with in the five years period (Fig. 6). A map showing overall picture of the five year outbreak report in the country is produced (Fig. 7).

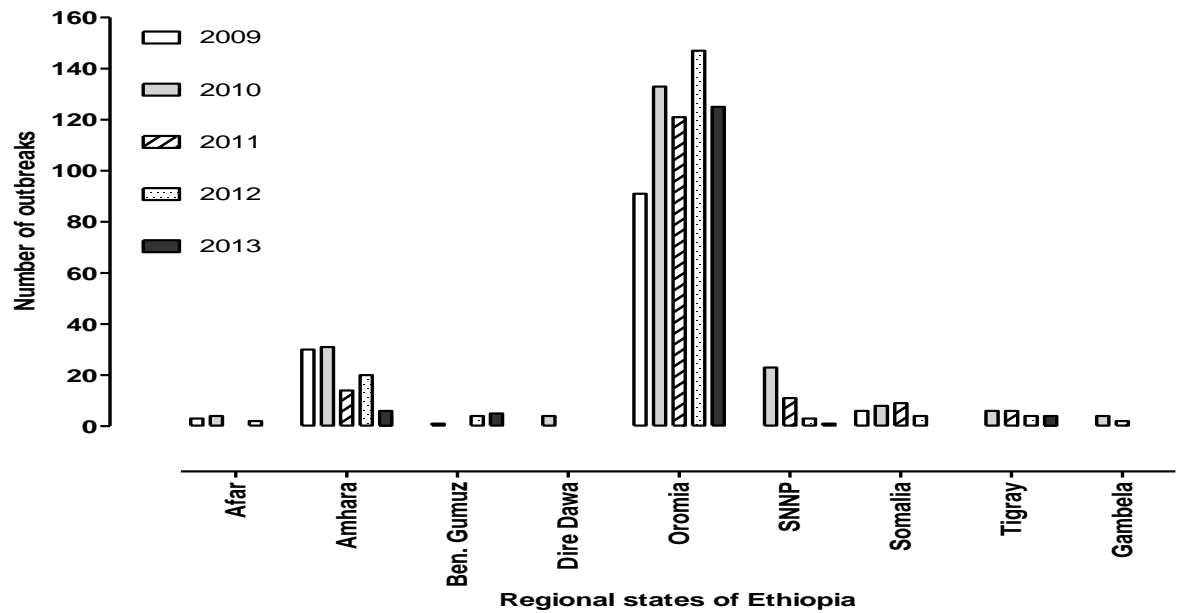


Fig. 6: Region wise number of outbreak during 2009-2013

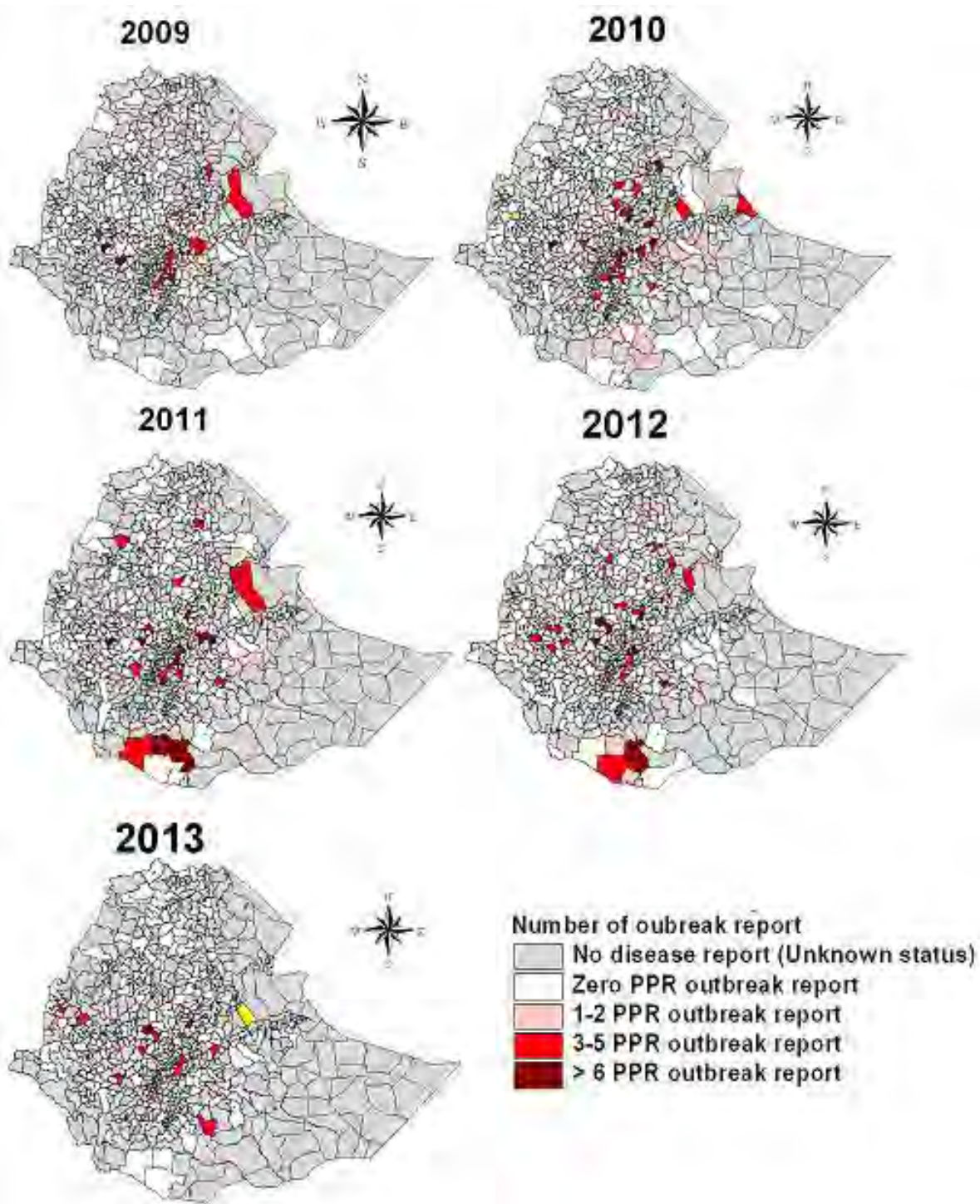


Fig. 7: Maps showing PPR outbreak report status of Ethiopia (2009-2013)

4.2. PPR clinical disease investigation through participatory approach

The clinical disease observations and outbreak investigation were done in 18 PA/villages of five districts (Raya kobo, Werebabo, Habru, Bati and Kewet). Both vaccinated and unvaccinated flocks were investigated for the presence of the clinical disease (PPR virus) and PPR antibody.

4.2.1. Community perception of PPR based on questionnaire interview

During the current participatory epidemiological study, different clinical symptoms were reported by livestock keepers in suspected PPR cases. The signs included nasal discharges, diarrhea, respiratory distress, oral ulcers and nodules, lacrimation and abortion (Fig. 8).

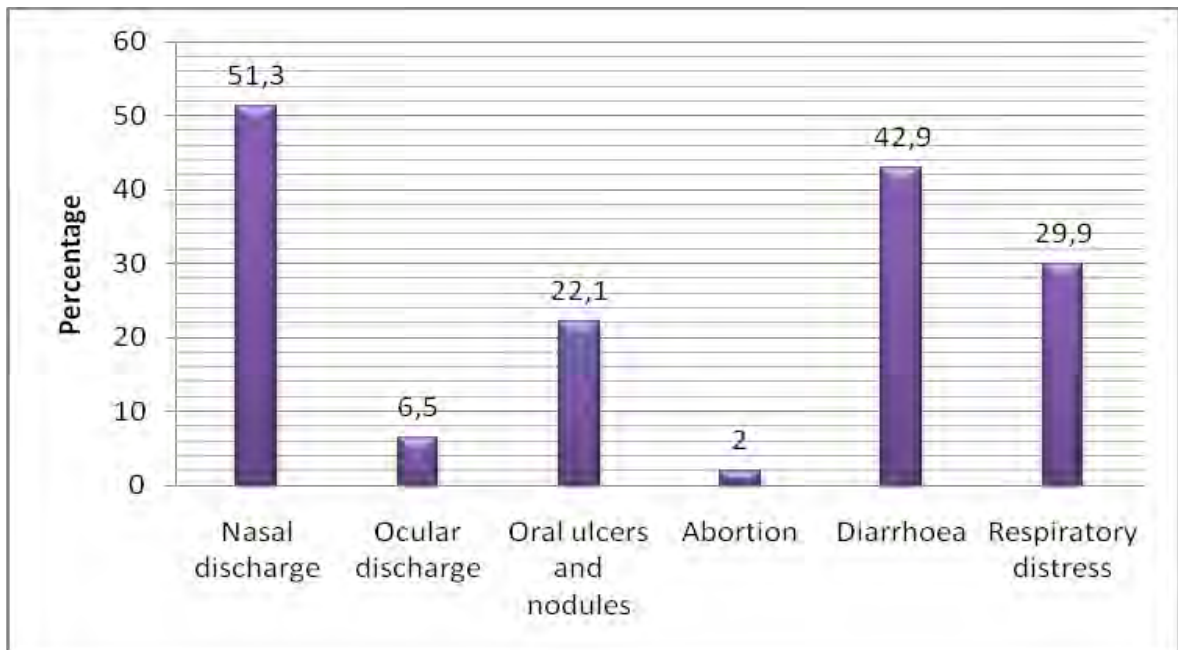


Fig.8: Suspected clinical symptoms of PPR as reported by respondents (N= 154)

4.2.2. Observation on clinical signs of PPR and quantifying its magnitude

Clinical signs of PPR were observed in both sheep and goats, in both sex and all age groups, in the all study districts. The clinical signs included high fever, ocular and nasal discharge, few abortions, respiratory distress and diarrhoea was also present in a few incidences in young animals. Besides the above clinical signs, erosive and necrotic stomatitis was also seen exceptionally in Habru district (Fig. 9).

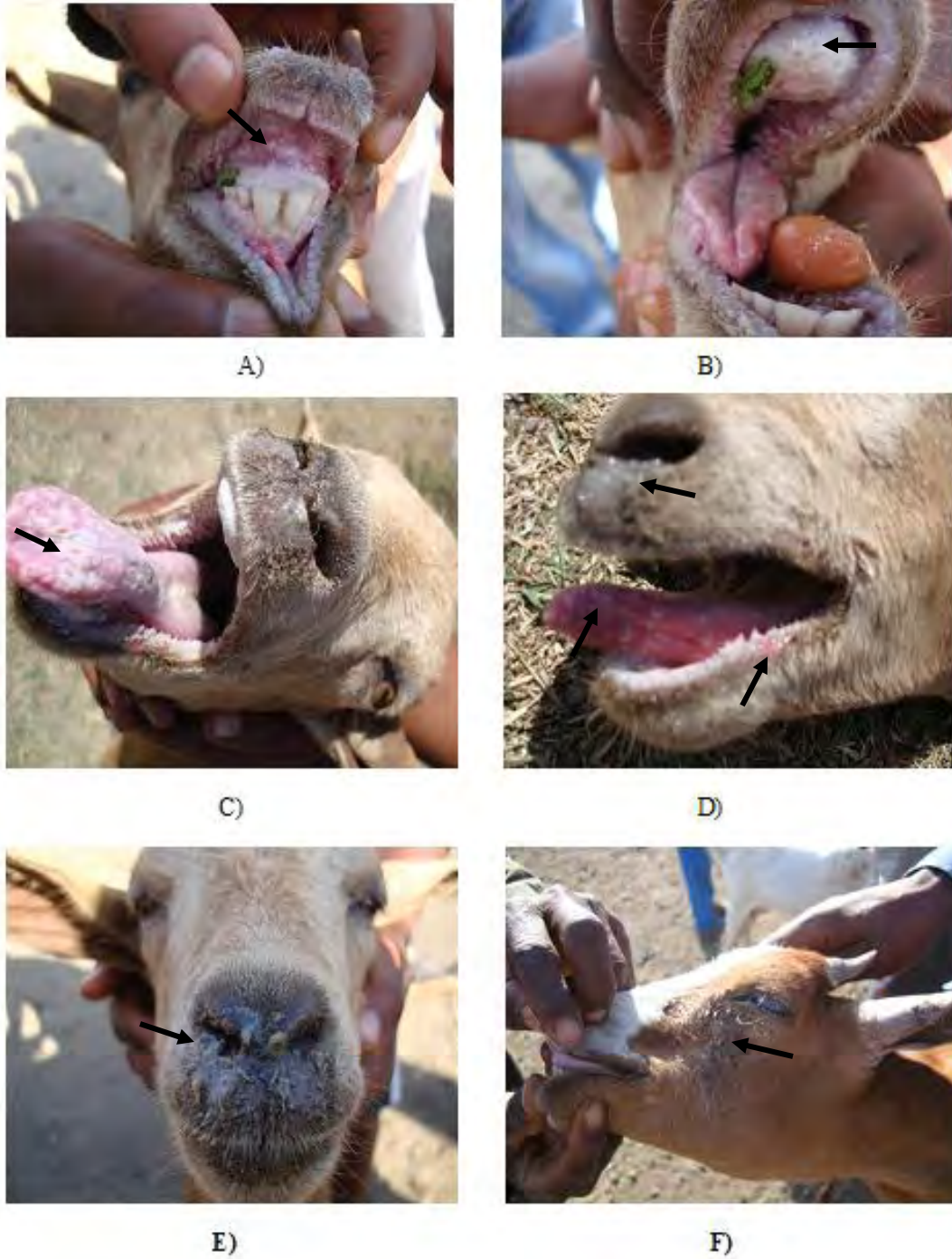


Fig. 9: Various clinical signs of PPR: A) Erosive and necrotic stomatitis and B) the upper dental pad completely obscured by a thick cheesy material C) Ulceration on the upper surface of tongue D) serious nasal discharge, dead cells on the surface of tongue and lesion on lower lip E) Muco-purulent nasal discharge and F) lacrimation

The disease outbreak was severe in Habru district and overt clinical signs were also observed.

The flock consisted of 121 sheep and 390 goats and could be regarded as homogeneous with respect to the risk of transmission of an infectious disease. Among this group under observation, there were 48 affected sheep and 64 affected goats giving morbidity rates of 39.7 and 16.4%, respectively. Nine sheep and 34 goats died of the disease with the mortality rates of 7.4 and 8.7%, respectively (Table 4). The case fatality rate was 18.8% for sheep and 53.1% for goats. The clinical signs and mortality rate were more severe in goats than in sheep. The course of the disease was reported to be between 4 and 10 days. In affected cases of the disease there was satisfactory response to injectable antibiotics as seen by treating animals during the outbreak.

Interestingly, the outbreak in Habru District was reported to be associated with the entry of newly purchased animals from a common ‘Mersa’ market. A complete history of the origin or the source of the animals to the market, whether from an area endemic for PPR disease, was not available.

Table 4: The mortality, morbidity and CFR during PPR outbreak in Habru district in 2014

Parameters	Sheep	Goat	Total
Population investigated	121	390	511
Morbidity	48 (39.7%)	64 (16.4%)	112 (21.9%)
Mortality	9 (7.4%)	34 (8.7%)	43 (8.4%)
CFR	18.8%	53.1%	38.4%

4.2.3. Pen-side test/ Lateral flow device

Though not validated for field use, there was promising result using the penside test (lateral flow device) on the spot in Habru district for the confirmation of the disease (Fig. 10).

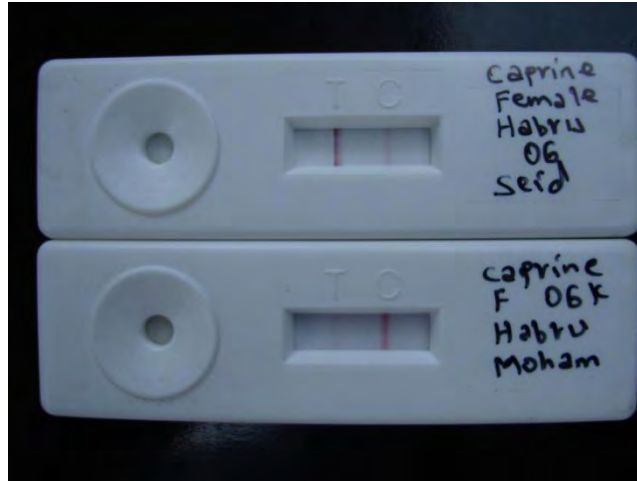


Fig. 10: PPR pen-side test/ Lateral flow device showed that the upper strip is strong positive and the lower one showed weak positive result

4.3. Serological studies

A total of 969 serum samples collected from the five districts were screened for specific antibodies against PPRV using c-ELISA kit. Of these samples, 612 (63.2%), 196 (20.2%) and 161 (16.61%) shoats were declared vaccinated, non-vaccinated and of unknown vaccination history, respectively by their owners and local veterinary personnel (Table 5).

4.3.1. Seroprevalence of PPR and associated risk factors in unvaccinated population

Prevalences

The overall seroprevalence of PPR virus antibody in unvaccinated small ruminants was 28.1% (95% CI = 21.9 - 34.9%) with highest (51.1%) and lowest (16.1%) seroprevalence in Bati and Habru districts respectively (Table 5), the difference which was also statistically significant ($p = 0.000$).

Table 5: Magnitude of seroprevalence levels among various vaccination statuses in small ruminants as compared among five Districts

Districts	Vaccinated					Unvaccinated					Unknown		
	N	Pos (%)	95% CI	χ^2	P-Value	N	Pos (%)	95% CI	χ^2	P-Value	N	Pos (%)	95% CI
Kobo	104	81 (77.9)	68.7-85.4	47.59	0.000	118	26 (22)	14.9-30.6	16.63	0.0002	-	-	-
Werebabu	98	73 (74.5)	64.7-82.8			-	-	-			-	-	-
Habru	179	88 (49.2)	41.6-56.7			31	5 (16.1)	5.5-33.7			-	-	-
Bati	176	104 (59.1)	51.4-66.4			47	24 (51.1)	36.1-65.9			-	-	-
Kewet	55	49 (89.1)	77.8-95.9			-	-	-			161	91(56.5)	48.5-64.3
Overall	612	395 (64.5)				196	55 (28.1)				161	91(56.5)	

Table 6: Prevalence of PPR antibodies in sera of small ruminants using cELISA

District/PA	Vaccination status	Sheep		Goat		Overall	
		No. sampled	Positive (%)	No. sampled	Positive (%)	No. sampled	Positive (%)
Raya Kobo							
Addis Alem	Vaccinated	9	9 (100)	48	48 (100)	57	57 (100)

Sodu	Vaccinated	-	-	47	24 (51.1)	47	24 (51.1)
Addis Kegn	Unvaccinated	33	7 (21.2)	30	7 (23.3)	63	14 (22.2)
Dibi	Unvaccinated	5	1 (20)	50	11 (22)	55	12 (21.8)
Overall	Vaccinated	9	9 (100)	95	72 (75.8)	104	81 (77.9)
Overall	Unvaccinated	38	8 (21.1)	80	18 (22.5)	118	26 (22)
Werebabo							
China	Vaccinated	16	13 (81.3)	31	16 (51.6)	47	29 (61.7)
Arebat	Vaccinated	19	18 (94.7)	32	26 (81.3)	51	44 (86.3)
Overall	Vaccinated	35	31 (88.6)	63	42 (66.7)	98	73(74.5)
Habru							
Tis Keye	Vaccinated	6	2 (33.3)	57	27 (47.4)	63	29 (46)
Jarota	Vaccinated	12	8 (66.7)	41	17 (41.5)	53	25 (47.2)
Mehal	Vaccinated	21	13 (61.9)	42	21 (50)	63	34 (53.9)
Amba							
Dire	Unvaccinated	14	1 (7.1)	17	4 (23.5)	31	5 (16.1)
Roka							
Overall	Vaccinated	39	23 (59)	140	65 (46.4)	179	88 (49.2)
Bati							
Chekorti	Vaccinated	14	8 (57.1)	44	30 (68.2)	58	38 (65.5)
Felana	Vaccinated	33	21 (63.6)	26	21 (80.8)	59	42 (71.2)
Chachatu	Vaccinated	27	13 (48.1)	32	11 (34.4)	59	24 (40.7)
Salemene	Unvaccinated	11	8(72.6)	36	16 (44.4)	47	24 (51.1)
Overall	Vaccinated	74	42 (56.8)	102	62 (60.8)	176	104 (59.1)
Kewet							
Kure	Vaccinated	20	19 (95)	35	30 (85.7)	55	49 (89.1)
Beret							
Sefi	Unknown	16	10 (62.5)	39	23 (59)	55	33 (60)
Beret							
Medina	Unknown	14	7 (50)	35	20 (57.1)	49	27 (55.1)
Yelen	Unknown	57	31 (54.4)	-	-	57	31 (54.4)
Overall	Unknown	87	48 (55.2)	74	43 (58.1)	161	91 (56.5)
Overall	Vaccinated	177	124 (70.1)	435	271 (62.3)	612	395 (64.5)
	Unvaccinated	63	17 (26.9)	133	38 (28.6)	196	55 (28.1)
	Unknown	87	48 (55.2)	74	43 (58.1)	161	91 (56.5)

Risk factors

Descriptive statistics for the variables under study and the results of univariate comparisons are presented in Table 7. The proportions of seropositive animals

significantly differ between districts, sex, age groups, grazing management, whether they introduce new animal or not and status of veterinary service.

There was no statistical difference in the seroprevalence recorded in goats (28.6%) compared to that in sheep (27%). The PPR seroprevalence recorded in female (23.1%) is significantly lower than the male (38.7%) animals sampled in this study. Age-wise, 34.7% of the adults and 9.6% of the young were seropositive, which was significantly differing at $p < 0.05$. Prevalences of PPRV infection was also seems varied among flock size, ranging from 27% to 44.4%, though it is not significant; however, these figures may not be accurate because of the small sample sizes in the Large flock (Table 7).

Those variables which significantly differ with Chi-square univariable analysis at $P < 0.05$ were further analyzed using the multivariable logistic regression model for risk factor analysis. The model revealed residing in Rayakobo district, adult age, communal grazing and recent introduction of new animals as risk factors for PPR seropositivity in sheep and goats (Table 8).

Table 7: Association of assumed exposure variables with seropositivity of PPR

Variables	Catagories/Levels	N	Seropositives (%)	95% CI	p-value
District	Raya Kobo	118	26 (22)	14.9-30.6	0.0002
	Habru	31	5 (16.1)	5.5-33.7	
	Bati	47	24 (51.1)	36.1-65.9	
Species	Sheep	63	17 (27)	16.6-39.7	0.8173
	Goat	133	38 (28.6)	21.1-37	
Age	Young	52	5 (9.6)	3.2-21	0.0005
	Adult	144	50 (34.7)	27-43.1	
Sex	Male	62	24 (38.7)	26.6-51.9	0.024
	Female	134	31 (23.1)	16.3-31.2	
Flock size	Small	122	33 (27)	19.4-35.8	0.5318
	Medium	65	18 (27.7)	17.3-40.2	
	Large	9	4 (44.4)	13.7-78.8	
Recent introduction of new animals	Yes	44	20 (45.5)	30.4-61.2	0.0019

	No	139	30 (21.6)	15.1-29.4	
Grazing management	Private	57	10 (17.5)	8.7-29.9	0.002
	Communal	96	40 (41.7)	31.7-52.2	
Raising type	Sheep and goat together	49	14 (28.6)	16.6-43.3	0.1527
	Sheep and goat separately	44	11 (25)	13.2-40.3	
	Mixed with other LS	60	25 (41.7)	29.1-55.1	
Feed shortage	Yes	76	26 (34.2)	23.7-46	0.6883
	No	77	24 (31.2)	21.1-42.7	
Shortage of Vet. Service	Yes	65	28 (43.1)	30.8-56	0.034
	No	83	22 (26.5)	17.4-37.3	

Table 8: Multivariable Logistic regression analysis of variables associated with seropositivity to PPR in non-vaccinated sheep and goats

Term	Odds Ratio	95% C.I.	Coefficient	S. E.	P-Value
District (Habru/Bati)	0.0000	0.0000->1.0E12	-17.4158	293.0887	0.9526
District (Rayakobo/Bati)	<u>0.1554</u>	<u>0.0439-0.5503</u>	-1.8617	0.6451	<u>0.0039</u>
Age (old/young)	<u>10.4924</u>	<u>2.3882-46.0978</u>	2.3507	0.7552	<u>0.0019</u>
Grazing management (communal /private)	<u>16.4103</u>	<u>4.5085-59.7317</u>	2.7979	0.6592	<u>0.0000</u>
Introduction of new animals (yes/no)	<u>17.1226</u>	<u>4.2963-68.2410</u>	2.8404	0.7054	<u>0.0001</u>
Sex (male/female)	0.5085	0.1747-1.4796	-0.6764	0.5450	0.2146
Shortage of Vet Service (yes/no)	1.9858	0.6725-5.8643	0.6860	0.5525	0.2143
CONSTANT	*	*	-8.0261	2.0842	<u>0.0001</u>

4.3.2. Seroprevalence and its assumed determinants in vaccinated population

Out of the 612 vaccinated small ruminants, the overall seroprevalence of antibody was 64.5% (95% CI = 60.6% - 68.3%) indicating the level of seroconversion in the five districts. There was statistically significant ($p= 0.000$) differences in seroprevalence

among the vaccinated districts (Table 9). Kewet district recorded the highest prevalence (89.1%) and Habru district with lowest prevalence (49.2%) in vaccinated area. At Kebele level, Addis Alem kebele turned 100% seroprevalence in Raya kobo district compared to the lowest seroprevalence (40.7%) in Chachatu Kebele in Bati district (Table 6). There was no difference in vaccine response of different age groups, sex, and species involved (Table 9).

Table 9: Association of assumed determinants/ fixed variables with sero-conversion of PPR antibody in vaccinated population

Variables	Catagories/Levels	N	Seropositives (%)	95% CI	p-value
District	Raya Kobo	104	81 (77.9)	68.7-85.4	0.000
	Habru	179	88 (49.2)	41.6-56.7	
	Werebabo	98	73 (74.5)	64.7-82.8	
	Bati	176	104 (59.1)	51.4-66.4	
	kewet	55	49 (89.1)	77.8-95.9	
Species	Sheep	177	124 (70.1)	62.7-76.7	0.0689
	Goat	435	271 (62.3)	57.5-66.8	
Age	Young	136	85 (62.5)	53.8-70.6	0.5339
	Adult	471	308 (65.4)	60.9-69.7	
Sex	Male	154	99 (64.3)	56.2-71.8	0.9386
	Female	458	296 (64.6)	60-69	

4.3.3. Determining the cut-off Percent of Inhibition (PI) values of the cELISA

The PIs pattern

The percent colour inhibition using cELISA provided an indirect measure of antibody levels in the test serum samples. Among the samples considered negative for PPRV (PI < 50%) the greatest number of samples had a PI of between 14 and 18. Alternatively, among the samples considered positive for PPRV PI ≥ 50% a peak frequency distribution of between 90% and 94% PI was observed (Fig. 11).

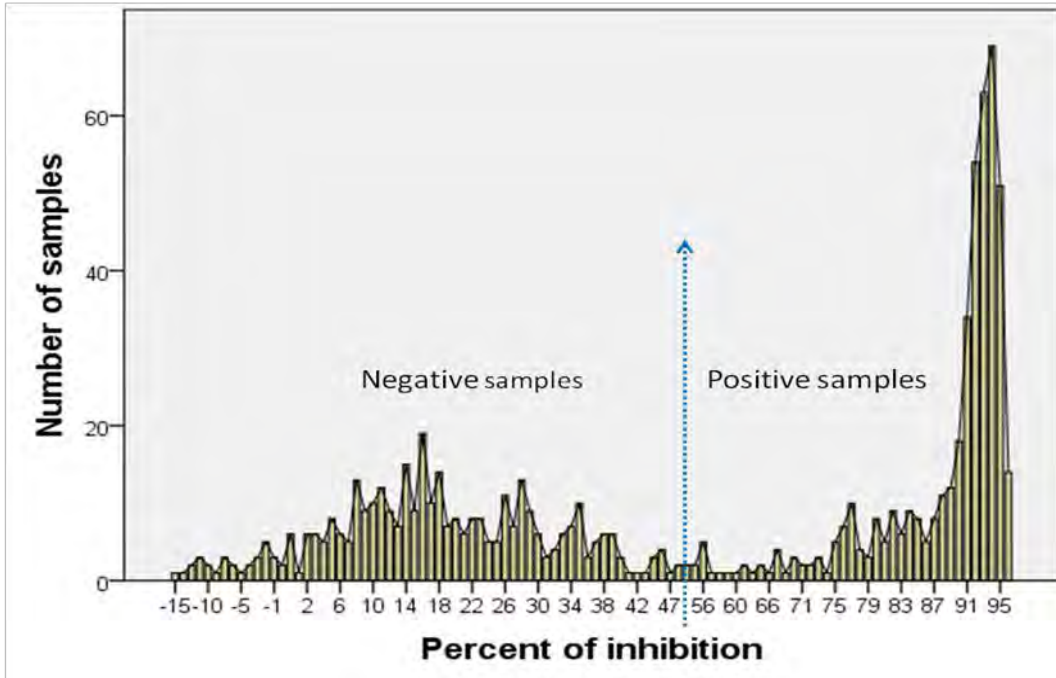


Fig. 11: Distribution of % of colour inhibition for PPR antibody positive and negative samples by cELISA, the arrow indicates the cut-off value for positive test sera (PI = 50%).

Comparative frequencies of the PIs between the vaccinated and unvaccinated groups

Considerable differences were observed between the vaccinated and unvaccinated populations when the results of the cELISAs were plotted as a frequency of the percent of colour inhibition (Fig. 12).

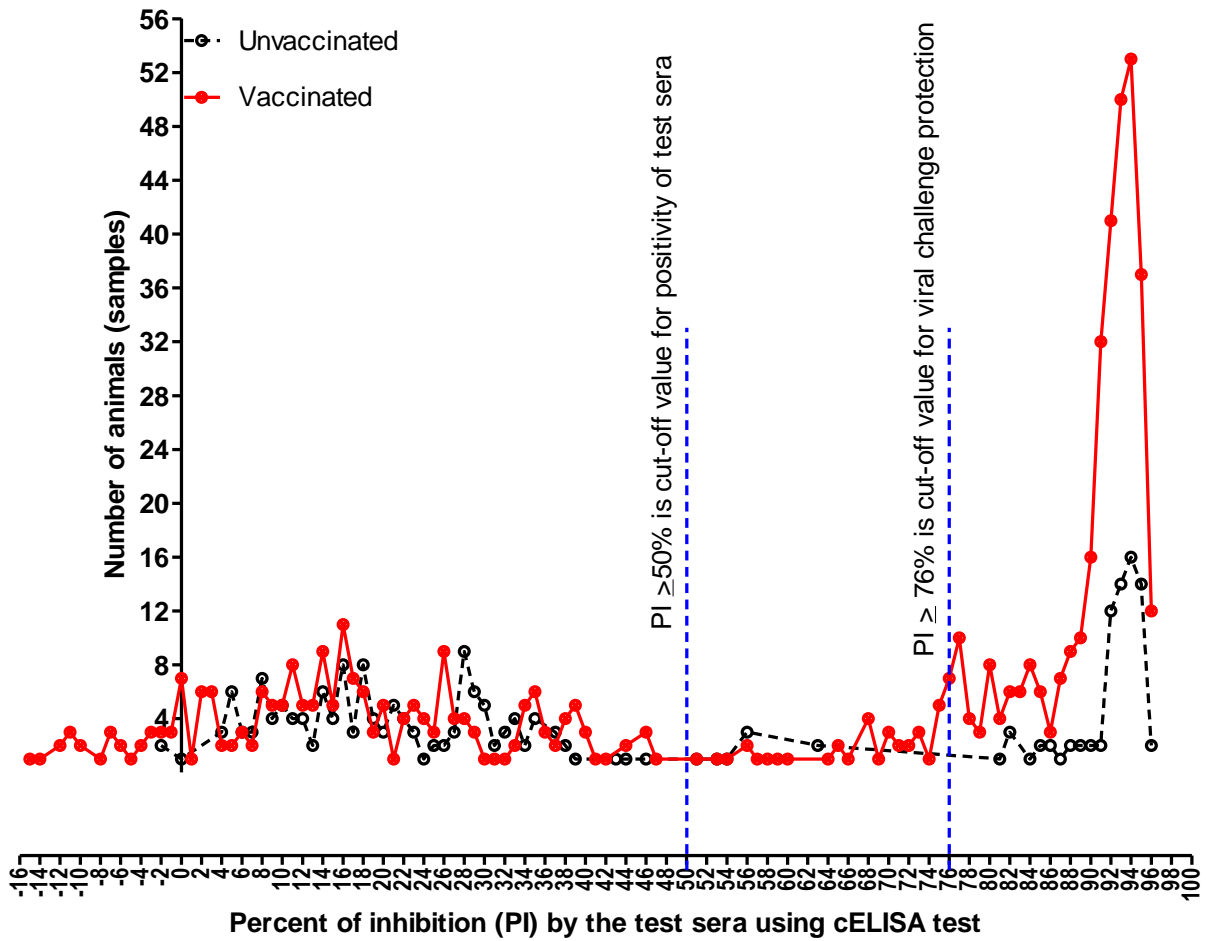


Fig. 12: Distribution of % of colour inhibition for vaccinated versus unvaccinated small ruminants along with cut-off value for detection of positive ($PI \geq 50\%$) and viral challenge protective ($PI \geq 76\%$) antibody titer in the test sera using cELISA.

In unvaccinated population around 28.1% and only 24.5% were with $PI \geq 50\%$ and 76%, respectively, whereas in vaccinated population around 64.5% and 58.8% of animals were with $PI \geq 50\%$ and 76%, respectively (Fig 13).

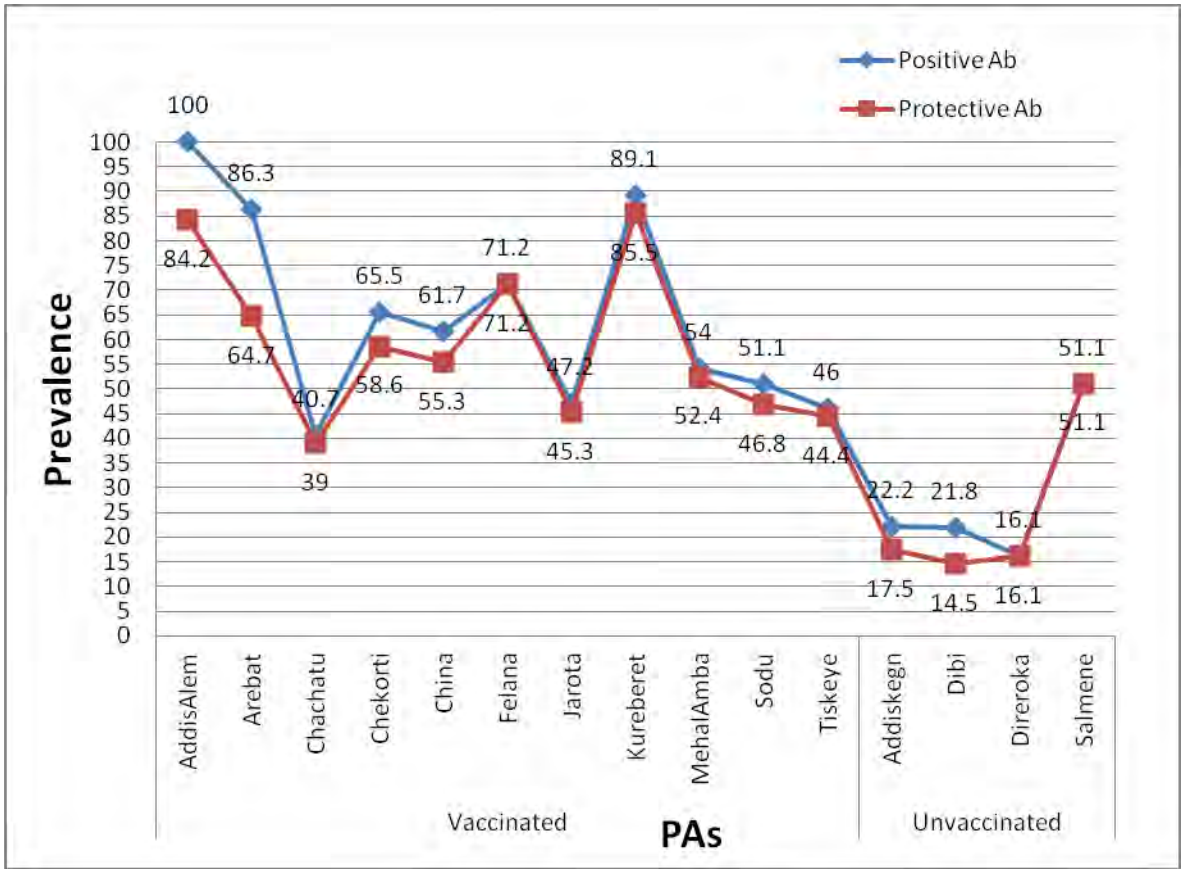


Fig. 13: Prevalence of PPR antibody for positivity and protection in vaccinated versus unvaccinated PAs

The median value of PI is 26 and 83 for unvaccinated and vaccinated population, respectively indicating the success of the vaccine administered as additional 57% of the population has gained protection due to vaccination (Fig. 14).

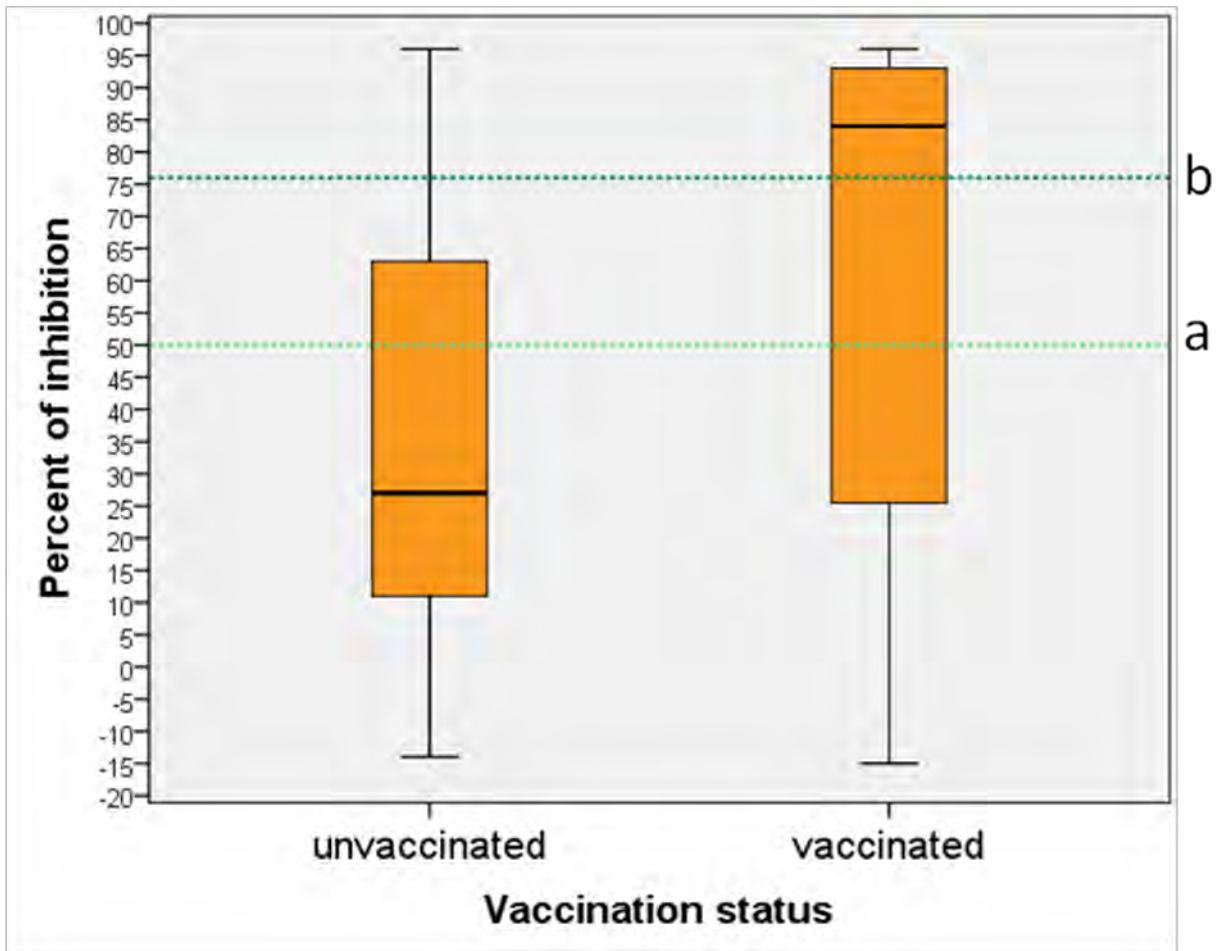


Fig. 14: Proportion of population (a) positive antibody titer for PPR ($PI \geq 50\%$) and (b) protective antibody titer for viral challenge ($PI \geq 76\%$) using cELISA in the vaccinated versus unvaccinated population.

4.4. Virus detection by I_c-ELISA, isolation on CHS-20 cell lines and confirmation using RT-PCR, and IFAT assay

4.4.1. Virus detection using IcELISA

Out of a total of 32 suspected samples (whole blood, nasal swab, ocular swab, buccal debris) examined with Ic-ELISA, 10 (31.3%) samples were positive for PPR viral antigen (Table 10). Highest presence of PPR virus was recorded in Raya kobo district, 6/6 (100%) followed by Habru district where it was found in 2/6 (33.3%) samples.

The species-wise disease outbreaks were more severe in goats than sheep as the incidence of viral antigen was 13.3 % in sheep as compared to 47.1 % in goats which is statistically significant (Table 12).

Table 10: Results of Ic-ELISA for detection of PPR antigen in suspected field samples

Districts	Type of samples	Ic-ELISA	
		N	Positive (%)
Raya kobo	Whole blood	6	6 (100)
Habru	Whole blood, nasal and ocular swab, buccal debris	6	2 (33.3)
Bati	Whole blood, nasal swab	20	2 (10)
Over all		32	10 (31.3)

4.4.2. Virus detection and confirmation using RT-PCR

From a total of 28 samples examined with RT-PCR for viral nucleic acid, 13 (46.4%) samples were positive (Table 11). Figure 15 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).

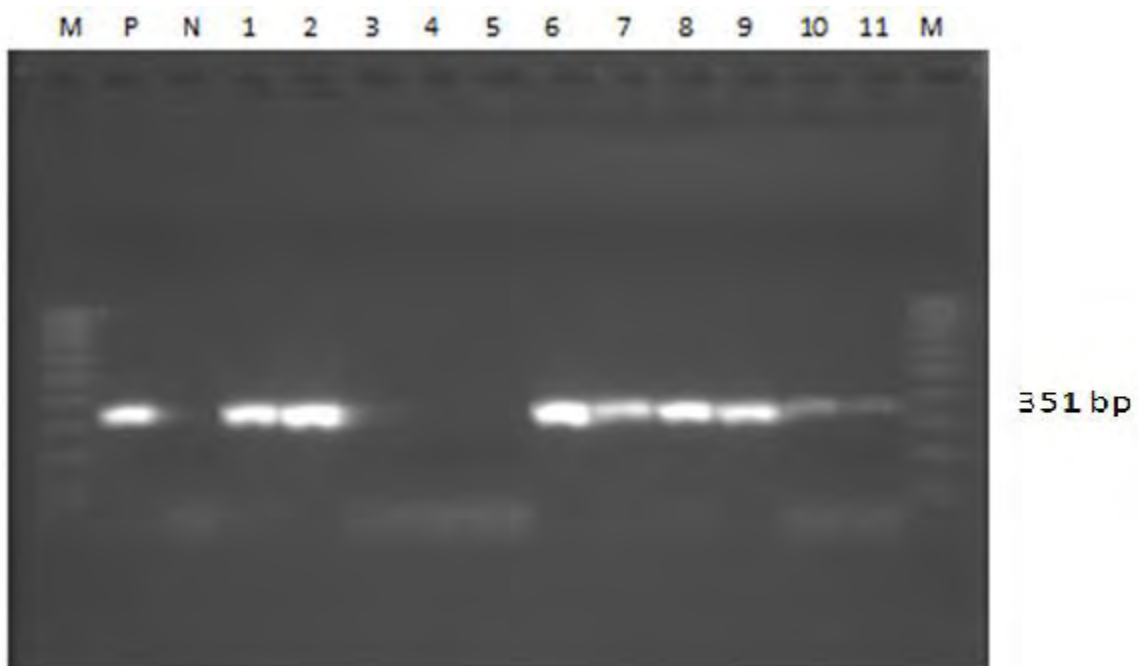


Fig. 15: Agarose gel electrophoresis of PCR products (351 bp) amplified with NP3 and NP4, PPR specific primers. Lane M: 100bp DNA molecular weight marker; Lane P: Positive control; Lane N: Negative control; Lane 1-11: Field samples.

Highest presence of PPR virus was recorded in Raya kobo district, 4/4 (100%) followed by Habru district where it was found in 3/5 (60%) samples examined with RT-PCR. The share of samples taken from each district is given in Table 11.

Similarly with Ic-ELISA, the species-wise disease outbreaks were more severe in goats than sheep as the viral nucleic acid was detected 21.4 % in sheep as compared to 71.4 % in goat. Statistically, there is significant difference in detection of PPR in sheep and goat at $p < 0.05$ (Table 12).

Table 11: Results of RT-PCR for detection of PPR viral nucleic acid in suspected field samples

Districts	Type of samples	RT-PCR	
		N	Positive (%)
Raya kobo	Nasal swab	4	4 (100)
Habru	Nasal and ocular swab, buccal debris	5	3 (60)
Bati	Nasal swab	13	6 (46.2)
Kewet	Nasal swab	6	0
Over all		28	13 (46.4)

Table 12: Species-wise detection of viral antigen and nucleic acid

Animal species	RT-PCR				Ic-ELISA			
	N	Positive (%)	95% CI	p-value	N	Positive (%)	95% CI	p-value
Sheep	14	3 (21.4)	4.7-50.8	0.0229	15	2 (13.3)	1.7-40.5	0.045*
Goat	14	10 (71.4)	41.9-91.6		17	8 (47.1)	23-72.2	
Total	28	13 (46.4)			32	10 (31.3)		

*Fisher exact test

4.4.3. Virus detection, isolation on CHS-20 cell lines and confirmation on IFAT assay

For this evaluation, the 18 samples from small ruminant in which PPRV RNA had been detected by classical RT-PCR were used. Out of the four pooled field samples, the PPR virus were successfully isolated on CHS-20 cell lines only from pool 4 collected from Habru district (Table 13). The CPE was detected on day 1 after infection and without any subsequent blind passage in CHS-20 cell culture. It was characterized by the appearance of vacuolated syncytia (Fig. 16) in the cell monolayer while no CPE was observed in the control cells. The syncytia increased in size in the second day to form large cell clumps that detached from the cell layer. The flask was frozen when the CPE covered about 70% of the cell layer. The presence of the virus in the medium was confirmed by collecting and testing of the cell culture supernatant by RT-PCR, Ic-ELISA and IFAT (Fig. 17).

Four subsequent blind passages were undertaken for the remaining three pooled samples. An aliquot of the cells was also collected at the time of the blind passage and tested for the presence of the PPRV RNA. While the release of the virus in the infected cell culture supernatant was effectively demonstrated for the pool 4 sample which had developed syncytia in the CHS-20 cells, virus could not be detected in the cell culture supernatant medium for the 3 remaining pools that did not develop CPE. The results obtained for all the samples that were tested are summarized in Table 13.

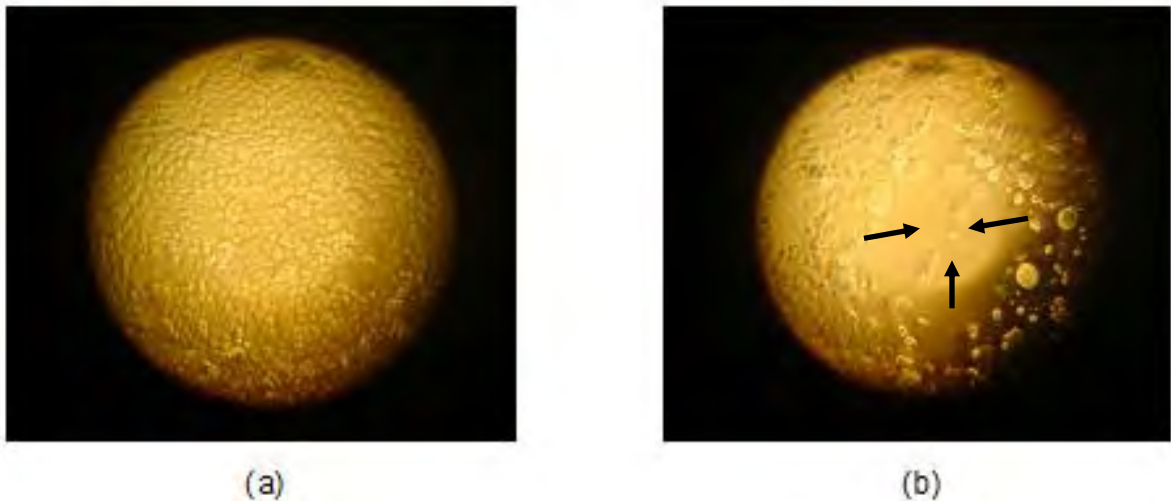


Fig. 16: Photographs of the microscopic observation of the CHS-20 cells: a) non infected cells, b) cells infected with pool 4 field samples showing rounding, aggregation, syncytia formation and destruction of the cell monolayer/pointed by the arrow (1 day post infection).

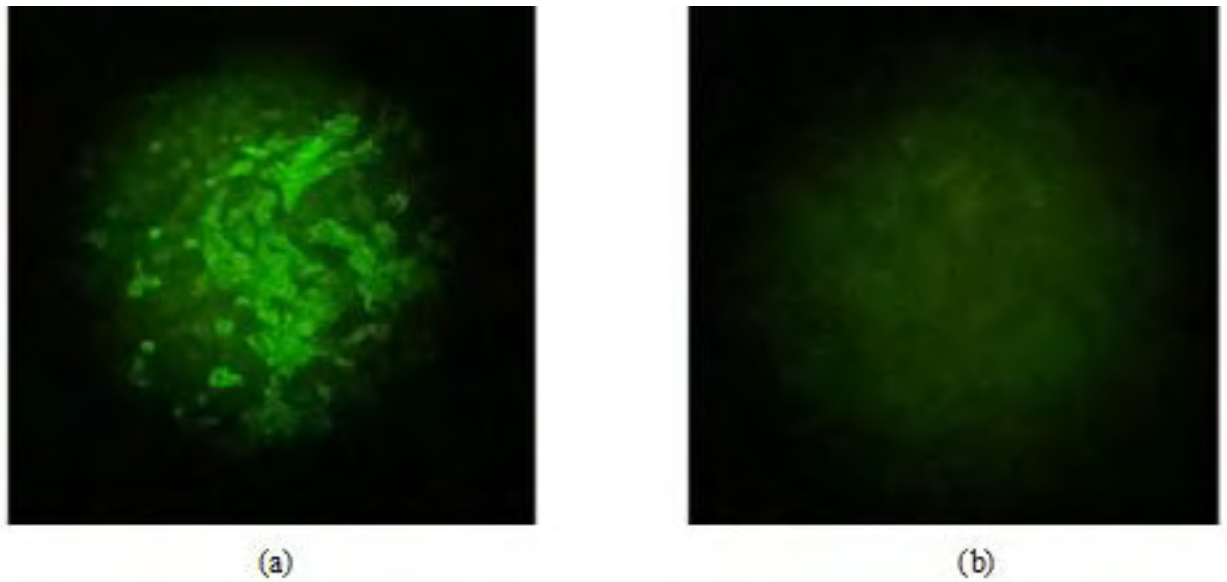


Fig. 17: Detection of the PPRV H protein by immunofluorescence staining, a) Positive culture b) Negative control, they were stained for indirect immunofluorescence with a PPRV-specific anti-H monoclonal antibody as previously described (Berhe *et al.*, 2003).

4.5. Test agreement of RT-PCR and Ic-ELISA and cell culture results of pooled samples

Eventhough 32 by Ic-ELISA and 28 clinical samples by RT-PCR had been examined; only 18 samples were commonly tested for PPRV by both Ic-ELISA and RT-PCR. Out of the 18 samples, 9 and 4 samples were positive by Ic-ELISA and RT-PCR, respectively. Six samples negative in Ic- ELISA yielded positive amplification in RT-PCR. Only one sample positive with Ic-ELISA found negative with RT-PCR. Thus overall agreement between Ic-ELISA and RT-PCR was 0.22 as shown in table 13.

Table 13: Test agreement of RT-PCR and Ic-ELISA and cell culture results of pooled samples

District	Village	Lab. code	Species	RT-PCR	Ic-ELISA	Pooled sample	CHS-20
Raya kobo	Addis Alem	33200	Caprine	+	ND	1	-ve
Raya kobo	Addis Alem	33201	Caprine	+	ND		
Raya kobo	Addis Alem	33202	Caprine	+	ND		
Raya kobo	Addis Alem	33203	Caprine	+	ND		
Bati	Felana	5141	Ovine	+	-	2	-ve
Bati	Felana	5142	Caprine	+	-		
Bati	Felana	5144	Caprine	+	-		
Bati	Chachatu	5148	Ovine	+	-	3	-ve
Bati	Chachatu	5149	Caprine	+	-		
Bati	Chachatu	5150	Ovine	+	-		
Habru	Tis keye	5154	Caprine	+	+	4	+ve
Habru	Tis keye	5155	Caprine	+	+		
Habru	Tis keye	5156	Caprine	+	+		
Bati	Felana	5143	Ovine	-	-		ND
Bati	Felana	5145	Ovine	-	-		
Bati	Felana	5146	Ovine	-	-		
Bati	Felana	5147	Ovine	-	-		
Bati	Chachatu	5151	Ovine	-	-		
Bati	Chekorti	5152	Caprine	-	+		
Bati	Chekorti	5153	Caprine	-	-		
Habru	Tis keye	5157	Caprine	-	-		
Habru	Tis keye	5158	Caprine	-	-		

Kappa value = 0.22, showing low test agreement between RT-PCR and Ic-ELISA; ND – Not done

5. DISCUSSION

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). Based on the reported morbidity and mortality of the infection and the flock size and structure of the small ruminant, it is likely that PPR became one of the most economically important livestock diseases in Ethiopia (Abraham, 2005).

Hence, an epidemiologically based targeting of endemic populations and high-risk zones is essential. The present study confirmed PPRV has been more recently circulating in village flocks of sheep and goats in the three of five studied districts existing in the low land highland interface (Raya kobo, Werebabu, Habru, Bati and Kewet) of Amhara region bordering Afar.

5.1. Spatio- temporal retrospective data analysis

Given that the weakness and inadequacy of the prevailing disease-reporting and surveillance systems in the country, the outbreak data documented in this study provide information about the endemicity of the disease that can help to formulate an effective strategy for a PPR-control programme in the country.

After the first confirmed cases of PPR in goats in Ethiopia (Roeder *et al.*, 1994), seasonal outbreaks were reported in many parts of the country (Abraham and Berhan, 2001). The current data shows clearly that the greatest number of outbreaks was observed after the main rainy season. This is in agreement with the study in India which indicates very few outbreaks in August and slowly increases from then until December (Hegde *et al.*, 2009).

The climatic factors may contribute to the survival and spread of the virus for the seasonal occurrence of PPR outbreaks (Abubakar *et al.*, 2011b). With the start of rains, the movement of animals is restricted due to the easy availability of local fodder. This may reduce disease transmission after the start of rains and during the period of easy availability of fodder (Abraham, 2005). Similar observations were also recorded in tropical humid zone of Southern Nigeria during a period of 5 years of observations (Taylor, 1984).

The spatial distribution also indicate that Oromia reported the highest number of outbreak followed by Amhara which may be attributed to the high population of small ruminants in these region in addition to the disease burden.

5.2. Epidemiological observations and disease investigation

This study reports PPRV has been more recently circulating in three districts of Amhara region bordering Afar. Previously, it is reported that infection with PPR virus is common in Ethiopia (Roeder *et al.*, 1994; Abraham and Berhan, 2001; Abraham *et al.*, 2005; Waret-Szkuta *et al.*, 2008; Megersa *et al.*, 2011; Delil, *et al.*, 2012).

The clinical findings, diagnostic investigation on samples collected from suspected animals as well as virus isolation consolidated the etiology of the disease to be PPRV. In general, the clinical features of PPR observed in the study districts are not different from those reported by others (Roeder *et al.*, 1994; Dhand *et al.*, 2002; El-Rahim *et al.*, 2005; Jindal *et al.*, 2005; Sharma *et al.*, 2007; Kul *et al.*, 2007; Khan *et al.*, 2007; Rita *et al.*, 2008; Abubakar *et al.*, 2009; OIE, 2013).

It seems that the severity of PPR outbreak in Habru district is much higher than the other districts since exceptionally an overt clinical signs of PPR were observed. In spite of the

fact that the village flocks had been vaccinated against PPR, the disease appears to be transmitted to few newly introduced animals. The outbreak involved of newly purchased sheep and goat from different market owned by recently returned youth from different Arab regions. The villagers testify that they did not experience any morbidity or mortality in their vaccinated flock. Only few the non vaccinated animals experienced the disease.

The overall morbidity, mortality and case fatality rate in sheep and goat in Habru were lower than the outbreak report in Egypt in kalubia province with 26.1%, 10.5% and 40.2%, respectively (Abd El-Rahim *et al.*, 2010). The highest overall mortality rate of 60% was estimated in a PPR outbreak in Ethiopian goats, while no mortality was observed in contact sheep. Similarly, Tibbo *et al.*, (2001) reported a higher morbidity of 76% and case fatality of 18% from a respiratory disease outbreak in sheep in central Ethiopia.

Mortality in susceptible flocks varies from 10 to 100% and morbidity ranges from 50 to 100%. However, this scenario is likely to change drastically once intensive vaccination programs are implemented for the target species (Balamurugan *et al.*, 2012).

5.3. Seroprevalence of PPR virus antibodies and associated risk factors in unvaccinated population

A diagnostic technique, which is simple, rapid, specific and sensitive is preferred for intensive surveillance of a disease. c-ELISA test is one such test for screening of antibodies to various morbilliviruses (Saliki *et al.*, 1993; Libeau *et al.*, 1995; Abd El-Rahim *et al.*, 2010; OIE, 2013). There was no information on the specificity and sensitivity of the PPR C-ELISA in the kit manual. Therefore, estimates by other laboratories that employed similar anti-N protein monoclonal antibody were accepted. The sensitivity of 93.4% and specificity 98.5% was reported (Choi *et al.*, 2005). The study showed that PPR was widely prevalent in small ruminants in the study areas. Given

the sensitivity and specificity of the test the results are likely to overestimate, slightly, the true proportion of seropositive animals (Singh *et al.*, 2004b; Choi *et al.*, 2005).

The overall seroprevalence (28.1%) in unvaccinated small ruminants was slightly lower than the finding in previous studies carried out in the country; 30.5% by Megersa *et al.*, (2011) but much higher than 6.8% by Abraham *et al.* (2005) and 6.4% by Waret-Szkuta *et al.* (2008). Comparable findings have been documented in other countries with the overall antibody responses to PPRV, 22.4% in Turkey by Özkul *et al.*, (2002); 33% in India by Singh *et al.* (2004a); 26% in Bangladeshi by Banik *et al.*, (2008); 32.8% in India by Balamurugan *et al.* (2012); 22.1% in Tanzania by Kivaria *et al.* (2013) and 34.2% in Pakistan by Munir *et al.* (2012a).

These results indicate that a certain percentage of small ruminants in the district have natural positive level of antibody titre against PPR. This could have probably resulted from field infection with PPR virus, which might protect against natural PPR challenge, as the humoral antibodies have shown to have a protective value in PPR (Bidjeh *et al.*, 1999, Islam *et al.*, 2003).

The inconsistency in the seroprevalence of antibodies to PPRV in different areas of the country is attributed to variations in a number of factors including the husbandry practice within different geographical regions, levels of immunity, diagnostic test, sampling procedures used and technical know-how of the researchers (Singh *et al.* 2004b; Waret-Szkuta *et al.* 2008).

The seroprevalence of 28.6% in goats and 27% in sheep in this study indicate a similar profile of serological status of the two species after PPR virus exposure which is in agreement with another study done by Taylor (1984) in the country. Similarly, Kivaria *et al.* (2013) recorded no association ($p = 1.000$) between animal species and seropositivity. Megersa *et al.* (2011) also reported comparable prevalence between the sheep (29.5%)

and goats (31.3%). This could be due to the fact that sheep and goats are equally exposed to the PPR risk factors such as movement and nutritional stresses, or it could be that the circulating field strain has overcome the natural resistance of sheep; different types of breed might affect the outcome of PPRV infection (Kivaria *et al.*, 2013).

Some authors (Gelagay, 1996; Ozkul *et al.*, 2002, Al- Majali *et al.*, 2008; Waret-Szkuta *et al.*, 2008; Swai *et al.*, 2009; Abubakar *et al.*, 2011c; Delil *et al.*, 2012) reported a higher seroprevalence in goats than in sheep and this is linked it to higher fecundity 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.

To the contrary, others reported higher seroprevalence in sheep than goats (Abraham *et al.*, 2005; Khan *et al.*, 2008; Saeed *et al.*, 2010). This was either related to a relatively lower number of sheep sampled in some of these studies or due to the fact that goats are often affected more severely by peracute and acute form of the disease, and might die prior to sampling.

A significant difference observed in seroprevalence of PPRV among study districts could be due to the variation in small ruminant population, animal health services, the movement of sheep and goat flocks for market as well as seasonal grazing and management system. Prior study by Waret-Szkuta *et al.* (2008) pointed out that there is large variation between regions and woredas of the country.

Khan *et al.* (2008) also reported that the higher numbers of positive cases were observed in southern and western districts of Punjab province, compared to other parts of the province. The northeastern parts of the province have got relatively small sheep and goat population, and, although previous outbreaks have been reported in this region following the transport of goats from endemic zones of the province (Athar *et al.*, 1995).

The differences observed in PPR seroprevalence between females and males was significant in this study whereby males were more affected compared to females. This is in agreement with Swai *et al.* (2009). This could be attributed to the fact that the high demands of male animals for meat purpose driven them to the market and contribute to the higher infection rate than in females which are relatively maintained at home for breeding purpose.

In contrast, Waret-Szkuta *et al.* (2008) and Khan *et al.* (2008) observed a significantly higher seroprevalence of PPR in females compared to males. This could be related to the physiological differences where females reveal some degree of predominance infection as a result of production and reproduction related stresses (Megersa *et al.*, 2011).

With respect to age category, the highest prevalence of PPR was observed in adults compared to young age. These findings are in agreement with previous reports from Ethiopia (Waret-Szkuta *et al.* 2008); Pakistan (Abubakar *et al.*, 2011); Turkey (Ozkul *et al.*, 2002) and India (Singh *et al.* 2004a); where they reported high prevalence in adults. It has been documented that sheep and goats exposed to natural infection to PPRV at a very young age may carry antibodies for 1-2 year following exposure and remains positive for a long time (Dhar *et al.*, 2002; Ozkul *et al.*, 2002; Singh *et al.*, 2004a).

In addition, communal grazing has significant association with the spread of PPR virus infection among sheep and goats than private grazing management in the study districts. Zahur *et al.* (2008) also reported an outbreak of PPR usually follows the introduction of new animals into the flock and the subsequent spread of the disease due to communal grazing. In agreement with this study, Abubakar *et al.*, (2009) reported continued year round circulation of the virus enhanced by frequent animal-to-animal contacts. Likewise, a study by Al-Majali *et al.* (2008) showed an association of large flock sizes and mixed farming with PPR seropositivity in Jordan.

Furthermore, shortage of appropriate veterinary services in the districts has also significant association with the higher seroprevalence of the PPR which could contribute to the wide prevalence and endemic establishment of the disease.

Few studies had addressed constant risk factors associated with seropositivity to PPR in Ethiopia. In this study, district, older age, communal grazing management and introduction of new animals appears to be a risk factor for seropositive status in the logistic regression analysis. This is in consistent with the findings of Abubaker et al. (2009), who reported a progressive increase of seroprevalence with increasing age. Similarly, introduction of new animals purchased from live animal market have been implicated as a source of the disease in India (Singh *et al.*, 2004a; Muhammad *et al.*, 2009).

In Jordan, large herd size was identified as a risk factor for PPR seropositivity in sheep and goat flocks and mixed (sheep and goats) farming was identified as a risk factor only in sheep (Al-Majali et al. 2008). Raising sheep along with goats was also found to be a risk factor for PPR seropositivity by other investigators (Anderson and McKay, 1994). Whereas visiting the live animal market was identified as a risk factor in India (Martrenchar *et al.*, 1995; Shankar *et al.*, 1988).

Targeting these factors in disease control programme may play a key role in limiting the transmission of PPR infection and augmenting effective disease control programmes.

5.4. Seroprevalence of PPR virus antibodies /herd immunity in vaccinated animals

In this study, herd immunity has been assessed by using c-ELISA and the antibody seroprevalence result indicated that herd immunity level against PPR was low in the

vaccinated area (64.5%) compared to a study done by Zahur *et al.*, 2009 in Pakistan indicated that the herd immunity threshold (HIT) for the flock, we need to achieve more than 85.4% vaccination coverage for control of PPR infection in sheep population. In Tanzania, Kivaria *et al.* (2013) determines that 63.8% of small ruminants in a population in agropastoral and 84.6% in pastoral farming system would have to be vaccinated with a 100 % efficient vaccine to reduce an effective reproductive number (R_t) value of 4 to less than 1, with the overall threshold level of vaccination necessary to eradicate the disease in small ruminants was 74.9%.

For rinderpest, HIT has been estimated as 75-80% (Rossiter and James, 1989). The herd immunity reported in Afar cattle vaccinated by CAHWs against Rinderpest was 83% (Mariner, 1996), which was greater than the current value for PPR. Similarly the post vaccination seroconversion study in small ruminant population of Awash Fentale District, Afar, Ethiopia conducted by Delil *et al.*, (2011) also recorded 61.13%, indicating relatively weak herd immunity. Luka *et al.*, (2011b) also noted an overall PPRV specific antibody sero-conversion of 55.3% among vaccinated small ruminants in Karamoja region, Uganda.

Comparison of Districts in terms of vaccination success showed that Kewet animals were more protected followed by Raya kobo, Werebabo, Bati district and Habru with the least sero-positivity. The sero-status suggests different level of vaccination coverage in the districts which has implication on the control of the disease.

The current low level of PPRV sero-positivity of vaccinated animals found in this study was unexpected since the PPR vaccine has been reported to confer protection for up to three years (Diallo *et al.*, 2007). Possible reason for this moderate to weak herd immunity could be poor announcement and awareness creation during vaccination campaigns that results in incomplete coverage of the population, or possibly faulty vaccination procedures. The antibodies due to natural exposure to PPR infection might also interfere

with the efficacy of vaccines. However, this scenario is likely to change if we include booster vaccination in the intensive vaccination programs to induce higher immunologic response for the target species.

5.5. Comparative protective antibody level of the vaccinated and unvaccinated population based on the PIs

The percence of colour inhibition indicated using a cELISA provided an indirect measure of antibody levels in the serum samples (Singh *et al.*, 2004) and it is highly correlated with VNT for end-point titration of PPR virus antibody (Libeau *et al.*, 1995). Findings suggest that the c-ELISA test developed can easily replace VNT for end-point titration of PPR virus antibodies (Singh *et al.*, 2004). In this study, thus, cELISA is used.

Only 24.5% of unvaccinated animals compared to 58.8% of vaccinated animal appeared to have protective PPRV specific antibody response (i.e. $PI \geq 76\%$). This indicates nearly a quarter of unvaccinated population and 60% of the vaccinated population have protective antibody. In other study, such antibody titers are completely protective from infection upon virulent PPRV challenge (Balamurugan *et al.*, 2012).

These findings suggest that only those animals capable of mounting a strong humoral antibody response to PPRV (high percent colour inhibition) were capable of surviving infection. This should not be confused with the higher prevalence of antibodies to PPRV (proportion of sera samples with a % colour inhibition of greater than 50%) observed in the vaccinated population versus the unvaccinated population (Singh *et al.*, 2004a).

If these preliminary findings on protective antibody ELISA titre in the vaccinated small ruminants are substantiated, they may have far reaching implications in evaluating the

immune status of national herd, which in turn tell about the level of vaccine coverage to better control the disease targeting wide vaccination coverage.

5.6. Detection of PPR virus in small ruminants

Detection of PPRV antibodies can confirm the diagnosis of PPR, however, in areas where specific vaccination against PPR is practiced, detection of PPRV antibodies may yield false picture of the prevalence of infection. Presence of maternal antibodies may further contribute to this problem. Thus, in such cases, detection of PPR virus in clinical samples becomes essential. Monoclonal antibodies have often been used to develop ic-ELISA, which is rapid and highly sensitive (Saliki *et al.*, 1994; Libeau *et al.*, 1995).

To confirm the presumptive diagnosis, immunocapture ELISA was used in this study for the detection of PPR viral antigen in 32 suspected samples. The results showed that 31.3% of samples were positive for PPRV antigen. This indicates that PPR virus was the causative agent of the outbreak and that endemic PPR virus is circulating within and between the small ruminant flocks. This can be compared to the findings of 40.98% by Abubakar *et al.* (2008); 21.4% by Munir *et al.* (2009); 34.3% by Abubakar *et al.* (2011); 25.7% by Munir *et al.* (2012) and 75% by Abd El-Rahim *et al.* (2010) who utilized the same IC-ELISA technique.

However, screening with RT-PCR appeared more sensitive and a total of 46.4% (13/28) samples were detected positive. Recently, Anees *et al.*, 2013 also found 25% (8/32) positive by the amplification of the nucleoprotein (N) gene. Nardi *et al.*, 2012 and Luka, *et al.*, 2011a confirmed the presence of PPRV in 33.3% (7/21) and 51.2% (17/33) clinical samples tested, respectively, using a set of primers specific for the F gene of the PPRV.

The current study also revealed a significant higher rate of infection in goat than sheep samples with both Ic-ELISA and RT-PCR. Abraham (2005) observed apparent absence of pathogenicity in sheep and explained this could result from a particular resistance of the local species and/or a loss of virulence of the Ethiopian PPRV strains for sheep. Similarly, Abubakar et al. (2008) reported that outbreaks of PPR in Pakistan were more severe in goats than in sheep. Mahajan et al. (2013) also noted a higher incidence of PPR infection in goats than sheep.

It is interesting to note that some of animals showing mild clinical signs were PCR positive however they were ELISA negative. The reason might be that the animals were in the early phase of the infection. For Rinderpest the severity of the clinical signs is correlated with the abundance of viral antigen in lymphatic organs and the mucosae along the digestive tract. It is likely that the same is true for PPRV (Diop *et al.*, 2005). Hence, the test agreement between the two tests was low. Previous study on a single PPR outbreak in Senegal revealed that for the West African long legged goat population, a peak of excretion could be detected at 1 week post infection, before the rise of antibodies at 14 days. It is also suggested that during the recovery period the infected animals are unlikely to be detected by antigen detection procedures (Diop *et al.*, 2005). It is observed that RT-PCR was able to detect virus secretion in ocular swabs at four days post infection (PI) in experimentally infected goats, as compared to eight days PI by IcELISA (Abubakar *et al.*, 2012).

Therefore, the rapid detection by suitable and appropriate methods of antigen and nucleic acid detection of PPRV in infected animals will help in early diagnosis of infection and subsequently control of the PPR disease in Ethiopia.

5.7. Virus isolation and confirmation

PPR virus should be isolated from field samples in cell culture for further identification, even when the detection of PPR viral antigen has been carried out by rapid immunocapture ELISA (Lefevre and Diallo, 1990; OIE, 2013). The current study revealed that the inoculation, isolation and propagation of PPR virus in CHS-20 cells was successful from the first passage in one of the four pool samples, with the CPE characteristic in concordance with that described by the World Organisation for Animal Health (Adombi *et al.*, 2011; OIE, 2013).

The presence of the virus in the CHS-20 medium was confirmed by collecting and testing of the cell culture supernatant by RT-PCR, Ic-ELISA and IFAT. Durojaiye *et al.* (1984) detected PPR viral antigen in tissue using FAT. Similarly, the virus was isolated in primary lamb kidney cells and identified by agar gel diffusion testing and immunocapture ELISA by Saeed *et al.*, (2004).

6. CONCLUSION AND RECOMMENDATIONS

Ethiopia developed a strategy for the progressive control of PPR that builds upon the lessons learnt from rinderpest eradication. A progressive control campaign based on repeated inoculation of all susceptible small ruminants is unaffordable to be implemented. Hence, an epidemiologically based targeting of endemic populations and high-risk zones will be essential.

Although there are few reports about the seroprevalence of PPR antibodies in different areas of the country, the clinical and molecular findings of this study confirmed the circulation of PPR virus among populations of sheep and goats in the study areas and prevalence in actual outbreaks situation, which should be kept in mind while deciding the vaccination strategy for the control of the disease.

This study showed varying antibody levels in the affected districts reflecting the infection and vaccination profiles of the herds. There was serological evidence of seroconversion to the vaccine and seroprevalence to the circulating virus suggesting the level of vaccine coverage which is not enough to achieve herd immunity should the disease strike again in the population.

The present study also has provided valuable information on the seasonal variation and geography of PPRV in sheep and goats from the past five years passive outbreaks data in the country. PPR occur throughout the year but is most prevalent through September and October, reaching a peak in November. The most appropriate time to vaccinate flocks is, therefore, in the months of August and September, thus budget release from MOFED should be appropriated.

In addition, the rapid detection by suitable and appropriate methods of antigen and nucleic acid detection of PPRV in infected animals will help in early diagnosis of infection and subsequently control of the PPR disease in Ethiopia.

In addition, the study has identified the source of introduction of PPR to be newly purchased animals and communal grazing, therefore, the first level of control is the restriction of movement of animals from endemic areas, with rigorous quarantine and surveillance procedures if a total ban is not practical to prevent the spread of the disease and the transmission of the virus to different localities.

Therefore, based on the above conclusive remarks the following recommendations are forwarded:

It seems an opportune time to begin extensive serosurveillance for PPRV in the country along with measurement of clinical survey in the enzootic parts, so that regions can be demarcated into endemic, infected and PPR-free zones. This will help in launching a comprehensive control programme for PPR in the country.

The current ongoing government efforts to perform PPR vaccination of goats and sheep for the control of the disease should be encouraged and applied more strictly and strategically to the delineated high risk zone using the homologous PPR vaccine that is recommended by the OIE.

In addition, strict serosurveillance and monitoring of PPR is recommended, together with uninterrupted vaccination of migratory flocks at the borders between districts or provinces or regions, for effective control of the disease.

For rapid diagnosis to enable a swift implementation of control measures, further validation of pen-side tests such as the lateral flow that can be performed without the need for equipments or technical expertise are highly desirable.

There should be further studies to identify the gene sequences and lineage of the PPR virus isolated in this study so that we could better understand the recent molecular epidemiology of the disease.

Further studies on disease status in the spatial and temporal trends events in the rest of the lowland highland interface of the country are required to define the epidemiology of PPR in these important areas so that to develop effective control strategies for PPR in large area of the country.

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8. APENDICES

Appendix I: Questionnaire

I. Study Location and Interviewee Detail

Date of collection _____

Code No. _____

1. Region _____ Zone _____
 District _____ Kebele _____ Specific
 name of place _____

<u>Total No of Shoats in District</u>	<u>Total No of Shoats in Kebele</u>
Sheep _____ Goat _____	Sheep _____ Goat _____
Total _____	Total _____
Exotic sheep _____ Goat _____	Exotic sheep _____ Goat _____

2. Geo-reference of Kebele. Long _____ Latit _____ Altit _____
 3. Owner's Name _____ Male [] Female []
 4. Number of Livestock owned

Cattle	Equines			Sheep	Goat	Others	Total
	Camels	Donkey	Horse				

II. The History of PPR Occurrence

1. List important health problems and symptoms that cause Sheep and goat mortality in your area? /It could be in local language/
2. Have you had enteritis-stomatitis syndrome in shoats of your flock? yes _____, No _____
3. Have you had PPR (Local name??) in your shoats? Yes _____ No _____
4. When did the disease commence in the area (Kebele)? Season _____ Mon _____
 year _____

5. Have you seen such outbreak in the area before this time, < 1yr__ 1-2 Yrs__2-3Yrs__ >3Years__

6. How frequent PPR reoccurs in the area? Don't Know __Every 1yr__ Every 2yrs__ >3yrs__

7. What measures are taken to prevent the above listed health problems?
 Traditional treatment [] Modern treatment [] Vaccination [] No treatment[]
 Other _____

8. What problems do you face when treating or vaccinating sheep in your area (rank them)?
 Lack of modern services/clinics [] Lack of drugs and vaccines []
 Transport/distance [] Other _____

9. How many animals had got sick and died due to PPR among the flock? _____

Clinical sick shoats spp	Sex	<3mon lamb/kid	Young	Adult
1.				
2.				
Died/slaughtered	Sex	<3mon lamb/kid	Young	Adult
1.				
2.				

10. Origin of PPR outbreak in the neighboring Kebele or district to your village _____

III. Flock Management

1. Do you move your shoats to other place for grazing seasonally? Yes /No
 If yes, when_____, where _____, how long did you keep them there_____?

2. Grazing and watering resource managements

<p>Grazing/watering mgt</p> <p>Communal_____</p> <p>Private _____</p> <p>Both_____</p>

<p>Farming System</p> <p>Sedentary_____</p> <p>pastoral_____</p> <p>Transhumant_____</p>

3. How do you raise your sheep and goat?
 Sheep and goat grazing separately [] Sheep and goat grazing together [] Sheep and goat grazing with other livestock [] Sheep and goat tethered feeding at home [] Other _____
4. Housing: Fenced stable _____; House barn _____
5. Have you bought new shoats or introduced new shoats since 3 months before the onset of the outbreak? Yes/No, If yes, origin of the shoats, number, sex and age?

6. Name and distance (in km) of livestock market frequently used and the known shoats trade route around their area. _____
7. Did you vaccinate your shoats for PPR? Yes _____ No _____.
 If yes when? Before 1 mon _____ 1-2 mons _____ >3mons _____
8. What is the common lambing/kidding season in which most of the animals born?
 June – September [] October – January [] February – May []
9. Did you encounter any critical season of feed shortage? Yes [] No [] If yes in which season _____

Appendix II: Procedure of Extraction of RNA from swab specimens using Qiagen® RNeasy Mini Kit

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 RNwasy 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 n 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.

Appendix III: 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 concentration	Final concentration	μl X 1 reaction
RNase-free water	/	7.5 μl
5X PCR buffer	1X	5 μl
dNTPS mix 10mM	0.4mM	1 μl
Q solution		5 μl
Primer forwarded NP3 (10 μm)	6 μm	1.5 μl
Primer reversed NP4 (10 μm)	6 μm	1.5 μl
Qiagen enzyme mix		1 μl
Total Volume		22.5 μl
RNA		2.5 μl
Final reaction Volume		25 μl

4. Aliquate 22.5 μl of the prepared master mix in to approximately labeled 0.2microcentrifuge tubes in PCR work station for master mix
5. Add 2.5 μl 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°C	95°C	94 °C	60°C	72°C	72°C	4
Time	30min	15min	30sec	30sec	1min	5min	infinity

7. 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

Appendix IV: 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	1xTBE-buffer	1% agarose	2% agarose
10x10cm	50ml	0.5gm	1 gm
15x10cm	75ml	.75gm	1.5gm
15x15cm	112.5 ml	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 55oc, add the required amount of Ethidium bromide 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 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.
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 Ethidium bromide (EtBr).
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.

Appendix V: Procedure of cell culture

I. Sample preparation

1. The swab is macerated well in the transport medium itself used for collection.
2. The resulting suspensions transferred to a centrifuge tube and centrifuge at 3000 – 5000 rpm for 20 min.
3. The supernatant is collected and ready to be inoculated in to the suitable cell lines.

II. Inoculation of A suitable monolayer cell with the collected supernatants

1. Select a monolayer cell culture with a confluence of >70% and remove the growth medium.
2. Wash the culture twice with PBS.
3. Inoculate the specimen suspension on the test flask and PBS with antibiotics and antimycotic (VTM) and incubate at 37° c for 60 min to allow the virus to absorb on to the cell culture.(1ml for 25cm² and 3ml for 75cm²tissue culture flask)

4. Add maintenance medium (MEM with 2% FCS) and incubate the flask at 37° for appropriate time, 3-7 days. Keep also control flasks without any specimen inoculums.
5. Observe for the effect of virus action.
6. It is essential that each material be passed in cell culture at least three times before declaring any specimen negative. The presence of viruses can be detected by observing cytopathic effect.