EPIDEMIOLOGICAL STUDY OF *PESTE DES PETITS RUMINANTS* IN GOATS AND SHEEP IN SOUTH KIVU, DEMOCRATIC REPUBLIC OF CONGO

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A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF DEGREE OF DOCTOR OF PHILOSOPHY IN APPLIED MICROBIOLOGY (VIROLOGY OPTION)

Department of Veterinary Pathology, Microbiology and Parasitology

UNIVERSITY OF NAIROBI

2018

DECLARATION

This thesis is my original work and has not been presented for degree in any other University

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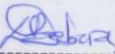
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DEDICATION

I dedicate this work to my parents Birindwa Ernest and Emmercianne Nabintu. A special gratitude to my loving wife, Charline Bwenge for the daily encouragement. My son, Nathan Ayagirwe and my daughter Nancy Asifiwe who have never left my side and are very special in my life.

ACKNOWLEDGEMENTS

I wish to sincerely thank the Rector of the "Université Evangélique en Afrique (UEA)", Prof. Gustave Mushagalusa Nacigera and the Dean of the Faculty of Agriculture and Environment Sciences of the Université Evangélique en Afrique (UEA), Prof. Katcho Karume for allowing me to take a study leave and acompagnied me with all kind of support that I could need.

My thanks are due to all colleagues who assisted me in one way or another in process of this study. Finally thanks to my family for their prayers; being patient, understanding and coping with problems that my study period may have imposed upon them particularly during the time that I have spent in Kenya while they are in Democratic Republic of Congo crossing fingers.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
μl	Microliter
AdF	Adenovirus expressing the PPRV F glycoprotein
AdH	Adenovirus expressing the PPRV H glycoprotein
AGDT	Agar Gel Diffusion Test
AGID	Agar gel immunodiffusion test
AGP	Agar Gel Precipitin
AMOVA	Analysis of Molecular Variance
ArcGIS	*Aeronautical Reconnaissance Coverage Geographic Information System*
B-ELISA	Blocking Enzyme-Linked Immunosorbent Assay
BLAST	Basic Local Alignment Search Tool
bp	Base pair
Bsm	*Bacillus smithii*
Bst	*Bacillus stearothermophilus*
BT	Bluetongue
CanL	Canine leishmaniasis
CaP	Capripox
CaPV	Capripox virus
cDNA	Complementary Deoxyribo Nucleic Acid
cELISA	Competitive Enzyme-Linked Immunosorbent Assay
CI	Confidence Interval
CIEP	Counter Immunoelectrophoresis
COAG	Council and the Committee on Agriculture
CPE	Cytopathic effects
DEPC	Diethyl pyrocarbonate
DIVA	Differentiation of Infected from Vaccinated Animals
D-loop	Displacement loop

DNA	Deoxyribo Nucleic Acid
DnaSP	Sequence Polymorphism
DRC	Democratic Republic of Congo
ELISA	Enzyme-Linked Immunosorbent Assay
EMPRES	Emergency Prevention System for Transboundary Animal and Plant Pests and Diseases
F	Fusion
FAO	Food and Agriculture Organization
GF-TADs	Global Framework-Transboundary Animal Diseases
GP	Goatpox
GPCR	G protein-coupled chemokine receptors
GTPV	Goatpox Virus
H	Hemagglutinin
HA	Haemagglutination Test
HI	Haemagglutination Inhibition Test
HV1	Hypervariable I region
IC-ELISA	Immunocapture Enzyme-Linked Immunosorbent Assay
IFAT	Immunofluorescent Antibody Test
IHC	Immunohistochemistry
IP	Immunoperoxidase Staining
L	Polymerase proteins
LSDV	Limpy skin disease virus
M	Matrix proteins
MEGA	Molecular Evolutionary Genetics Analysis
mRNA	Messenger RNA
mtDNA	Mitochondrial Deoxyribo Nucleic acid
NCBI	National Centre for Biotechnology Information
ng	Nanogramme
NP	Nucleoprotein

NPV	Net Present Value
OD	Optical density
OIE	Office International of Epizooties
OR	Odds ratio
P	Phosphoproteins
PCR	Polymerase Chaine Reaction
pfu	Plaque-forming units
pH	*Potential of hydrogen*
PIT	Precipitinogen Inhibition Test
Pmol	Picomol
PPR	*Peste des petit ruminants*
PPRV	*Peste des petit ruminants virus*
qRT-PCR	Quantitative reverse transcriptase polymerase Chain reaction
RNA	Ribo Nucleic Acid
RP	Rinderpest
RP030	RNA polymerase subunit gene
RPV	Rinderpest Virus
RT-LAMP	Reverse transcription-loop mediated isothermal amplification
RT-PCR	Reverse transcriptase Polymerase Chaine Reaction
SADC	Southern African Development Community
S-ELISA	Sandwich Enzyme-Linked Immunosorbent Assay
SNP	Single Nucleotide Polymorphism
SPPV	Sheep pox Virus
TADs	Transboundary Animal Diseases
TCRV	Tissue culture rinderpest vaccine
USD	United States Dollars
VEROc	African Green Monkey Kidney cells
VNT	Virus Neutralisation Test

ABSTRACT

Peste des petits ruminants (PPR) is an acute, contagious transboundary disease of sheep, goats and some wild (Gazelles, Springbuck, Impala) animals caused by *Peste des petits ruminants virus* (PPRV). The disease was first officially reported in Democratic Republic of Congo (D.R. Congo) with devastating losses since 2008. Since then PPR has spread to almost all pastoral counties in Eastern of D.R.Congo where goats and sheep are playing major role in farmer's livelihood. The control of PPRV in D.R. Congo have been limited due to lack of epidemiological information, including spatial prevalence distribution, genetic nature of the circulating virus, host-associated risk factors and socio-economic effects. Several pathogens including capripoxvirus (CaPV), *Pasteurella multocida* (PM), *Peste des petits ruminants* virus (PPRV), *Mycoplasma capricolum* and *capripneumoniae* (Mccp) are responsible for major respiratory syndromes of goats and sheep in D.R. Congo. The dual or multiple infections caused by several pathogens increase the morbidity and mortality rates within animals in flocks or between animals individually. *Peste des petits ruminants (PPR)* reduces the activation of the immune response to capripox in goats and sheep in a mixed infection resulting in increase of the mortality and morbidly rates and causing economic loss to farmers. The lumpy skin disease virus (LSDV), goatpox virus (GTPV) and sheeppox virus (SPPV) are species of Capripox genus.

The objectives of this research were to assess the status, prevalence, coinfection with capripox, associated risk factors and socio-economic impact of *Peste des petits ruminants* in sheep and goats and investigate the historical demographic dynamics of goats from PPRV outbreaks through mitochondrial DNA *D-loop* control region in South Kivu.

For prevalence of PPRV specific antibodies using competitive enzyme-linked immunosorbent assay (cELISA), 320 serum samples from both unvaccinated and asymptomatic goats and/or sheep were collected randomly from four different teritories of South Kivu, while associated infection determinants and socio-economic impacts of the disease were assessed by participatory interview through a structured questionnaire and farmer groups . The ArcGIS software was used to draw all PPR sero-prevalence maps. The molecular epidemiology of PPR virus (PPRV) targeting the conserved PPRV nucleoprotein (NP), fusion (F) and hemagglutinin (H) proteins was determined by Reverse-transcriptase Polymerase Chain Reaction (RT-PCR) using NP, F and H specific primers. A total of 150 samples including tissues, swabs and whole blood were collected from goats with PPR clinical signs, for virus characterization. Further, due to diagnostic constraints the same samples were used to check for coinfection with Capripox virus (CaPV) targets the *P32, RPO30* and *GPCR* genes using conventional One-step PCR. Both positive PPRV and CaPV samples were sequenced using Sanger method for further phylogenetic analysis using both CLC Genomics work bench version 10 and Molecular Evolutionary Genetics Analysis (MEGA) version 6 software. Genomic DNA of 111 indigenous goats from both *peste-des-petits ruminants virus* susceptible and non-susceptible varieties was extracted and the mitochondrial control regions (*D-loop* region) were amplified and sequenced with specific primers. Additional 22 goat mtDNA Hypervariable I region reference sequences (HV1: 481bp) belonging to the six known haplogroups/lineages were also downloaded from GenBank and included in the r analysis.

Results of PPRV sero-status and associated risk factors in South Kivu showed an overall PPRV sero-prevalence of 45.3% (n=320) from which 53.4% (n=240) was found in goats and 21.3 % (n=80) in sheep. A multilevel nominal logistic model using JMP-Pro software and Stata 11 indicated that the likelihood of goats and sheep being infected with PPRV increased significantly when the following occurred (p<0.05): animals shared water sources (p=0.003628), herd size increased (p=< 2.2e-16), animal age increased (p=2.630e-07), exotic breeds were used (p=0.005), animals were raised with goat flocks (p=0.0177310), animals were reared in communal grazing systems (p=0.0001004), different goat species were raised together (p=0.0073387), and when there was exchange of animals between farms (p=<2e-16). The overall molecular prevalence with RT-PCR was 64.7% (97/150). The molecular characterization revealed clustering of the PPRV within lineage III. Significant substitutions in the nucleoprotein (NP) gene sequences were observed.

Live animals demonstrated the following clinical signs: serous to mucopurulent nasal and ocular discharge, fever, diarrhea, lacrimation, matting of eye lids, cutaneous nodules; one may also see self-resolving lips and muzzle lesions of the involved animals. Other lesions seen on dead animals, on post -mortem examination were: congestion of lungs haemorrhages in large intestine and liver. Samples taken for laboratory diagnosis included: oculo-nasal swabs, tissues, whole blood and serum.

The results showed mixed infection between PPR and Capripox which emphasized on the importance of molecular techniques in differentiating the two diseases. Out of 150 tested animals, 64.7% (97/150) were PPRV positive, 52.7% (79/150) were Capripox positive and 38.7%

(58/150) were both PPRV and CaPV positive. The pair-wise comparison of P32 gene of CaPV and F gene of PPRV showed 99.75% of identity percentage among CaPV sequences, 96.95% among PPRV sequences and 47.91% of nucleotide percentage identity between CaPV and PPRV.

For the goat's mtDNA analysis, a total of 120 segregating sites, 56 haplotypes and 124 mutations were found in a 1,220-bp sequence. The mean haplotype diversity and nucleotide diversity were 0.971 ± 0.007 and 0.01068 ± 0.00206, respectively with the overall number of nucleotide differences of 10.731. The phylogenetic analysis showed that all goat sequences were clustered into two haplogroups A and B, of which haplogroup A was the commonest. The global analysis of molecular variance (AMOVA), indicated that 83.22% of the total genetic variation of studied animals was described by genetic dissimilarities between individuals (pv= 0.32658), 11.18% among groups (pv=0.0000*) and only 5.60% of the variation was attributed to genetic changes between goat populations (pv=0.00286*).

Briefly, the high seroprevalence in unvaccinated animals indicates that PPRV is circulating in South Kivu. Several risk factors are associated with PPRV sero-status including spatial and farm management. Peste des petits ruminants virus (PPRV) obtained from Eastern D.R. Congo clustered genetically with PPRV strains of Lineage III from East Africa, including Tanzania, Uganda and Kenya. This study informs the transboundary importance of this disease in the region.

The lumpy skin disease virus, which is one of the species of capripox genus; found in co-infection with PPRV in South Kivu is a lineage which is circulating and has a genetic relationship between its P32 gene of Capripox virus (CaPV) and the PPRV fusion gene.

There are high levels of intrapopulation diversity in Mwenga-Shabunda, Fizi and Kalehe goats and the weak phylogeographic structuring, suggesting the existence of solid gene flow between goat populations probably due to extensive trans-border goat's movement in the past from countries that have reported previous outbreaks of the PPR disease.

South Kivu pastoralist community recognised PPR among the economic disease affecting the goats and sheep and it has the potential of disrupting cultural set up and local economy with the daily economic losses due to morbidity rate associated with suspected PPR of 30.2 USD for sheep and 37.05 USD for goats and approximatively 10.26 USD for sheep and 120.72 USD for goats due to mortality rate.

It is thus recommended that protective effective measures should be applied in South Kivu including animal control movement within and outside the country in order to control the spread of PPR from the infected regions to non-infected areas. Vaccines that protect animals for both PPR and capripox are recommended for a better protection. Some goat breeds resistant to PPR can be selected and used in breeding programs.

CHAPTER ONE: GENERAL INTRODUCTION

1.1 BACKGROUND INFORMATION

Agriculture is one of the key sectors of the sustainable economy of Democratic Republic of Congo (D.R. Congo) and the means of livelihood for most of its rural population with emphasis that over 70% of the populations are living in the rural area (IPAPEL, 2016). Livestock production has been identified as a useful development tool with benefits that meet the aims of the Sustainable Development Goals (SADC, 2012a; FAO, 2016). Livestock keeping is the main source of livelihood for most pastoral households. Much as it is an important industry across Africa, the constant presence of disease is its biggest constraint; the OIE reports that 12 out of the 15 transboundary livestock diseases considered to be the most contagious are found in Africa. This burden of disease is further worsened by emerging infectious diseases. Such animal diseases are termed transboundary animal diseases (TADs), and one such disease that is attracting attention especially in Sub Saharan region is *Peste des petits ruminants* (SACIDS, 2012 b and c).

Peste des petits ruminants (PPR) is presently reflected as one of the major transboundary diseases of livestock causing high impact to farmers of developing countries (Banyard *et al.*, 2010). It is a highly infectious disease of small ruminants which has been reported in almost 70 countries in Africa, the Middle East and parts of Asia (Luka *et al.,* 2012). The disease has been estimated to cause losses worth USD 1.5 to 2 billion every year. In Democratic Republic of the Congo, the annual direct loss due to PPR, i.e. costs of dead animals is estimated at 5.3 million USD (SADC, 2012c; FAO, 2015). According to GF-

TADs Steering Committee for Africa, during the years 2008-2011, a total of 4,079 outbreaks, 431,258 clinical cases and 56,663 deaths due to PPR were reported in 25 to 30 (56%) countries in Africa but there is still lack of information on serological or molecular aspects in some parts (SADC, 2012b). *Peste des petits ruminants* morbidity and mortality rate goes up to 90 and 100%, respectively (Rossiter and Taylor, 1994; Rossiter 2004). Moreover, SADC (2012b) reports that, from 2010-2012, a total of 120,000 animals (goats and sheep) have died in D.R. Congo due to PPR. It is, therefore, estimated that around 600,000 sheep and one million goats are at risk of getting PPR, indicating one-quarter of goats and two-thirds of sheep in the whole country.

1.2 JUSTIFICATION OF THE STUDY

Peste des petits ruminants (PPR) disease has caused massive losses on livestock assets of the Congolese pastoral communities. The status of PPR in African great lake region including Rwanda, Burundi and D.R. Congo, which share borders and practice uncontrolled animal trans-border movement, is not well understood since there are very limited available studies that have been conducted to elucidate the situation of the disease in the region. In Eastern of D.R.Congo where this study was done, no studies have been done to diagnose and confirm the presence of the disease, using the molecular test; neither have studies been done to determine the genetic nature of the PPR virus strains circulating in South Kivu; while since 2008, the FAO survey reported cases of PPR based on the clinical signs (FAO, 2012). It is, therefore, important to understand the factors that have made the disease cross over to D.R. Congo and spread throughout the pastoral parts so that effective control measures can be designed and

applied. Misdiagnosis and problems related to differentiation of Capripox (CaP) from PPRV in the field might increase the mortality and morbidity rate in small ruminants and lead to the failure of the disease eradication and control programs. This study also assessed the genetic diversity of goats at mitochondrial DNA (*D-loop*) region to determine the origin and dynamics of animals towards disease patterns. Eradicating PPR or reducing its transmission is keys to reduce poverty in South Kivu, because goats and sheep are considered as the major investment of poor farmers. Goats and sheep are species of choice for pastoralists in D. R. Congo (around 80% of farmers) (Report from IPAPEL, 2016). Because of their lower cost and being easy to handle, many families use small ruminants to start rebuilding herds. Goats and sheep are also more readily marketed than large ruminants and are often slaughtered for home consumption, as large ruminants cannot be consumed by the family before spoilage.

1.3 OBJECTIVES

1.3.1 General objective

To assess the epidemiological and molecular status of *Peste des petits ruminants* in goats and sheep in South Kivu province of Democratic Republic of Congo.

1.3.2 Specific objectives

1. To determine the PPRV seroprevalence and its associated risk factors in South Kivu province of Democratic Republic Congo.

2. To carry out genetic characterization of the PPR virus isolated and establish the maternal lineages of indigenous goats from a PPR outbreak in D.R. Congo.

3. To assess the dual infection status of peste des petits ruminants and capripox and establish their molecular relationship at respectively F and P32 genes.

4. To estimate the direct economic losses occasioned by outbreaks of PPR based on perceived loss of benefits experienced by the goats and sheep farmers in South Kivu.

1.4 HYPOTHESES

i. There is high prevalence of PPR disease that is precipitated by several determinants in South Kivu province.

ii. *Peste des petits rumiants* virus of Lineage III is circulating in South Kivu as it's the stains that was commonly detected in neighboring countries of Est Africa and different genotypes of indigenous goats portray varying susceptibilities to PPRV strains that are circulating in South Kivu

iii. Coinfections of *Peste des petits ruminants* and Capripox viruses in South Kivu interfere with respective diagnoses as the two might be genetically related at some genes.

iv. Peste *des petits ruminants* inflict direct financial losses to small ruminant owners as a result of animal deaths and reduced production.

CHAPTER TWO: LITERATURE REVIEW

2.1 DISEASE DEFINITION AND HISTORY

Peste des petits ruminants (PPR) caused by peste-des-petits ruminants virus is endemic in D.R. Congo and many other Sub Saharan Africa countries (Banyard *et al.*, 2010) with goats being more severely affected than sheep (CFSPH, 2008; FAO, 2012). The disease was reported for the first time in 1942 in Côte d'Ivoire (West Africa) by Gargadennec and Lalanne (Diallo *et al.,* 2007). The disease has currently reported and confirmed in many countries of Africa, Middle East, Central and South Asia as well as in China (Munir *et al.,* 2013; Libeau *et al.,* 2014). *Peste-des-petits ruminants* has had diverse designations like "kata", "pseudo rinderpest", "syndrome of stomatitis-pneumoenteritis" and "pneumoenteritis complex" (Abdalla *et al*., 2012). The "plague" reference name of the disease indicate that PPR is highly contagious with high economic impact. Several authors among them, Hamdy *et al.* (1976) and Taylor (1979 a and b) reported that it is only during 1970s that PPR was distinguished from rinderpest virus using biochemical, cross protection and serology experiments. *Peste-des-petits ruminants* is categorized as a highly contagious critical or sub-acute febrile and often fatal disease of small ruminant (Furley *et al*., 1987; Braide, 1981). The disease is described by serous nasal and ocular discharges which eventually become mucopurulent (Zhao *et al.*, 2010). Ismail and House (1990) found that animal suffering from PPR is presented high fever, pneumonia, plentiful diarrhea and lameness and intestine and scabs around lips and cutaneous nodules have been also found in animals with peste des petits ruminants (Rossiter, 2004). Moreover, Merck Sharp and Dohme (2009) and Elsawalhy *et al.*(

2010) demonstrated PPR cases characterized with stomatitis, gastroenteritis, pneumonia and conjunctivitis, causing serious economic losses in the production of small ruminants.

2.2 THE *PESTE DES PETITS* RUMINANTS VIRUS

2.2.1 History and Classification

Peste des petits ruminants virus was isolate for the first time in cell culture by Gilbert and Monnier in 1962. Chauhan *et al*. (2009) classified PPR to *Paramyxoviridae* family, *Mononegavirales* order and *Morbillivirus* genus. This genus shares almost the same genetic features with causative agents of other highly infectious diseases (Bailey *et al.*, 2005). Among these diseases the most important include: rinderpest virus, canine distemper virus, measles virus, porpoise and dolphin distemper viruses that infect marine mammals (**Figure 2.1**). Due to seroconversion of cattle, in the past, PPRV was considered to be a variant of the rinderpest virus that had adapted to goats and sheep and lost the virulence for cattle (Gargadennec and Lalanne, 1942).
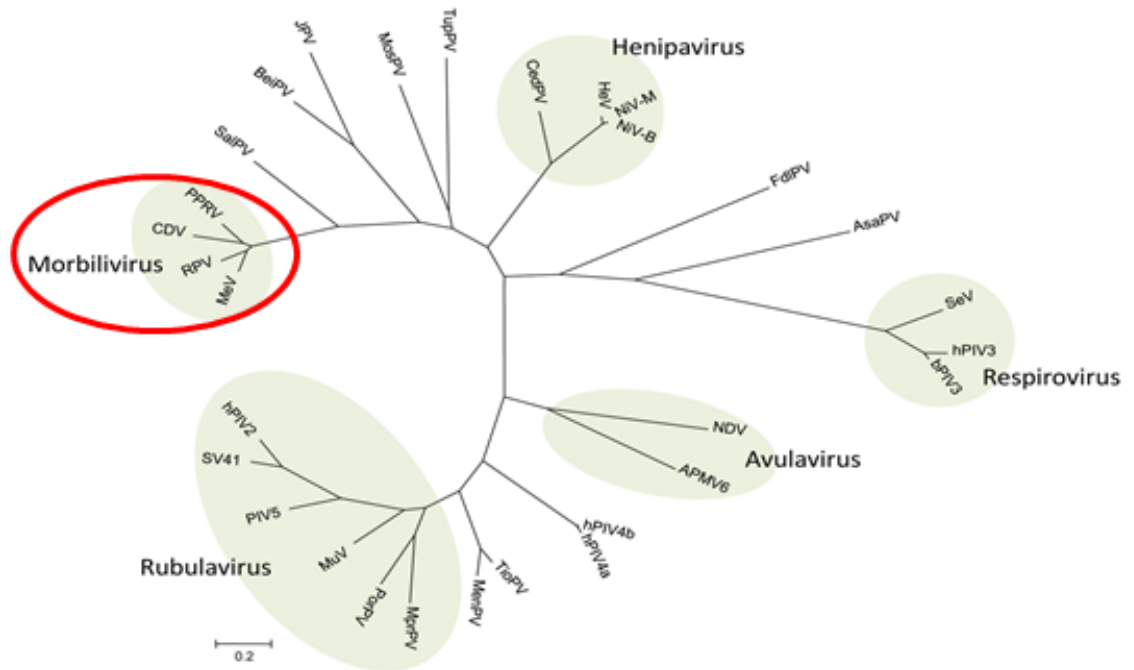
Figure 2.1: Phylogenetic tree based sequences on the NP protein of selected family paramyxoviruses showing Morbillivirus genus (Red Circle).

2.2.2 Structure

2.2.2.1 Physical structure

Viral particles of PPRV are enveloped spherical structures with fusion proteins (F) and hemagglutinin proteins (H) appearing as spikes on the virion surface (Fenner *et al.*, 1993). Matrix proteins (M) inside the envelope stabilize virus structure with the nucleocapsid core composed of the genomic RNA, nucleoproteins (NP), phosphoproteins (P) and polymerase proteins (L) (Barrett *et al*., 2006) (**Figure 2:2**). The nucleocapsid (N), which is associated to two other viral proteins: P and L, covered the viral RNA (Lefèvre and Diallo, 1990). The Matrix (M) protein make a link between

N and the glycoproteins F and H, which are responsible, for the attachment and the entry of the virus into the host cell respectively (OIE, 2008).
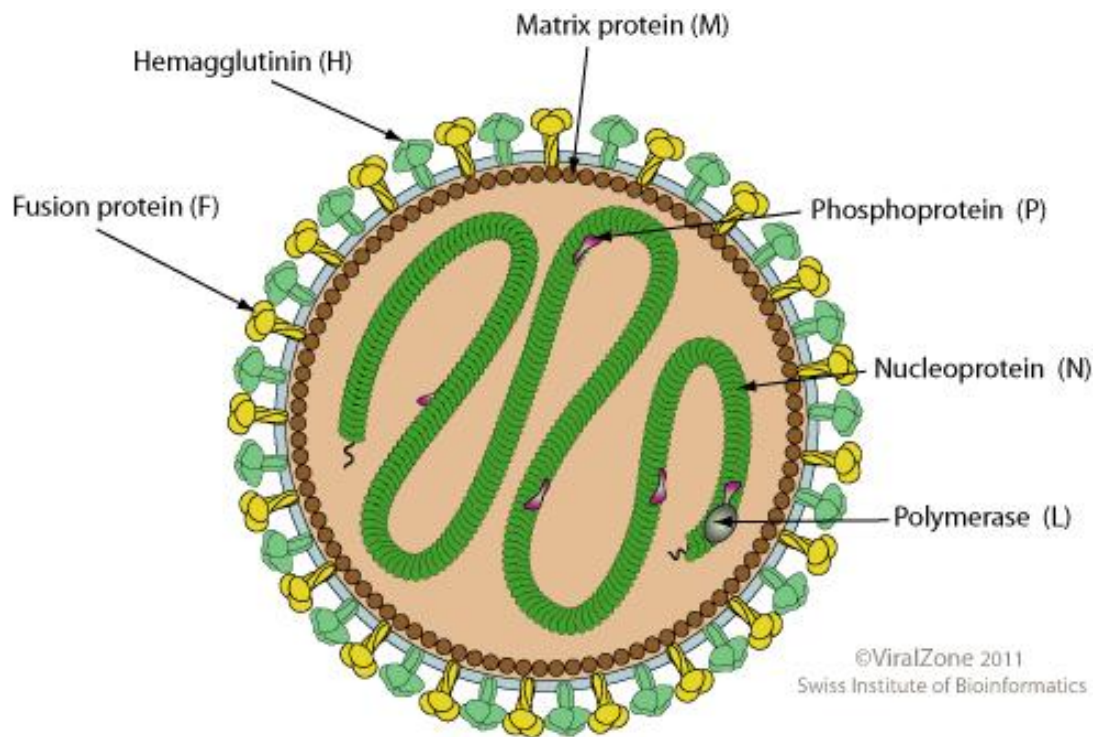
Figure 2.2: *Peste des petits ruminants* **virion genome organization**

2.2.2.2 Orientation

Like other *Morbilliviruses*, PPR virus is a non-segmented negative sens single-strand RNA o enveloped of 15,948 nucleotides (Qin *et al.*, 2012). The six structural (N-P-M-F-H-L) and two non-structural (C-V) PPRV genome proteins are found on the order of 3′-N-P/C/V-M-F-H-L-5′ on the genome flanked by 3′-Leader and 5′-Trailer responsible for the synthesis of positive and negative sense RNA. The P gene encodes for two non-

structural proteins, C and V, via alternative open reading frames and RNA editing (**Figure 2.3**).

Figure 2.3: Genome orientation of PPR Virus (Naveen *et al.,* 2014).

2.2.3 Survival

Peste-des-petits-ruminants virus has a cover/capsid derived from the host-cell plasma membrane with two transmembrane glycoproteins surrounding a nucleocapsid. The virus is also sensitive to ultraviolet radiation, heat and desiccation. Several studies have shown the PPRV thermal sensitivity (Rossiter and Taylor, 1994; Diallo, 2000). However, it was proved that PPRV survives in lymph nodes at 4°C for 8 days (Lefèvre, 1982). Moreover, the virus is sensitive to low pH environment but stable within a pH range between 5.8-9.5. At the room temperature, pH above 11 and below 4 the activity of virus can be loses rapidly (Diallo, 1990). Dufour (2010) showed that the best pH for PPRV persistence ranges from 7 and 8.

2.2.4 Life cycle

The life cycle of *Peste-des-petits-ruminants* virus begins with the adhesion of haemagglutinin to receptors of cell-surface before the fusion of the virion envelope with cellular membranes, then the release into the cytosol of infected cell of the virus nucleocapsid (Chauhan *et al.,* 2009). The virus polymerase enzyme binds to the single

promoter positioned at the 3' end of the genome (Barrett *et al*., 2006). The virus partially uncoats the nucleocapsid and transcribes the genes into positive-stranded mRNAs which are translated into non-structural and structural proteins (Lefèvre and Diallo, 1990). Transcription either terminates after a gene or continues to the next gene downstream, which means that genes close to the 3' end of the PPRV genome are transcribed in large quantities compared to those toward the 5' end which are least likely to be transcribed; this phenomenon is well-known to be transcriptional gradient (Cann, 2005). Kwiatek *et al.* (2010) proved that the nucleoprotein is the most expressed gene because of its location at the 3'end of the PPR viral genome. Therefore, the N concentration in the cell determines when the L switches from gene transcription to PPRV genome replication (Barrett *et al*., 2006). Replication results in full-length, positive stranded antigenomes that are in turn transcribed into negative-stranded virus progeny genome copies (Cann, 2005). Newly synthesized structural proteins and genomes self-assemble and accumulate on the cell membrane, bud off from the cell. The viral entry by binding of the HN protein to receptors is due to the interaction of the H, N and F PPRV proteins with the host plasma membrane (SLAM and other undisclosed receptors) for PPRV (Munir *et al*., 2013) **(Figure 2.4).**
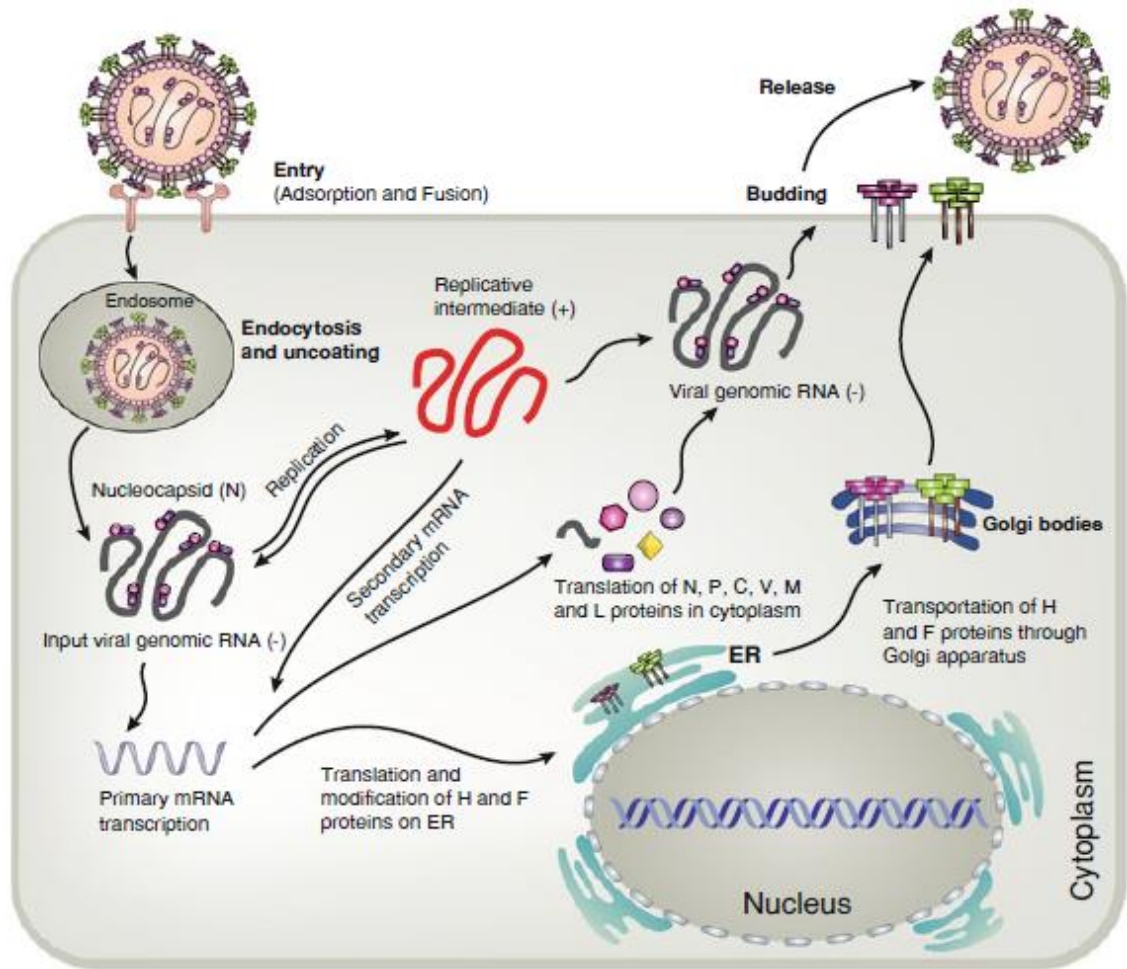
Figure 2.4: A generalized morbillivirus replication cycle

2.3 EPIDEMIOLOGY OF THE DISEASE

2.3.1 Molecular Epidemiology

The current PPRV isolates molecular characterization subdivides the PPR viruses into four genetically distinct lineages (I, II, III and IV) based on partial or full sequence analysis of F (**Figure 2.5)** and N (**Figure 2.6**) as suggested by Esmaelizad *et al.* (2011), Sharawi and Abd-El-Rahim (2011) and Luka *et al.* (2011 and 2012) and Kgotlele *et al.*

(2014). In one hand, the PPRV-lineage I is found mainly in West Africa since 1970s and recent in Central Africa; on the other hand the lineage II in West Africa especially in Burkina Faso, Ivory Coast and Guinea. Lineage III isolates have been more reported in Sudan and Eastern Africa while lineage IV was more observed in the Middle East and southern Asia, Arabian Peninsula and recently it has spread across several African countries (Banyard *et al.*, 2010; Parida *et al.,* 2016) (**Figure 2.7**).

The assessment of worldwide animal movement and the PPPRV classification into lineages has become easy with the evolution of molecular epidemiology (Balamurugan *et al*., 2010 and 2014). Lineage IV has been distinct to Asia even though it has been observed in north, west, central east and southern Africa thereby becoming the most widely circulating lineage according to several references among them Banyard *et al.* (2010), Kwiatek *et al.* (2011) and Luka *et al.* (2012).
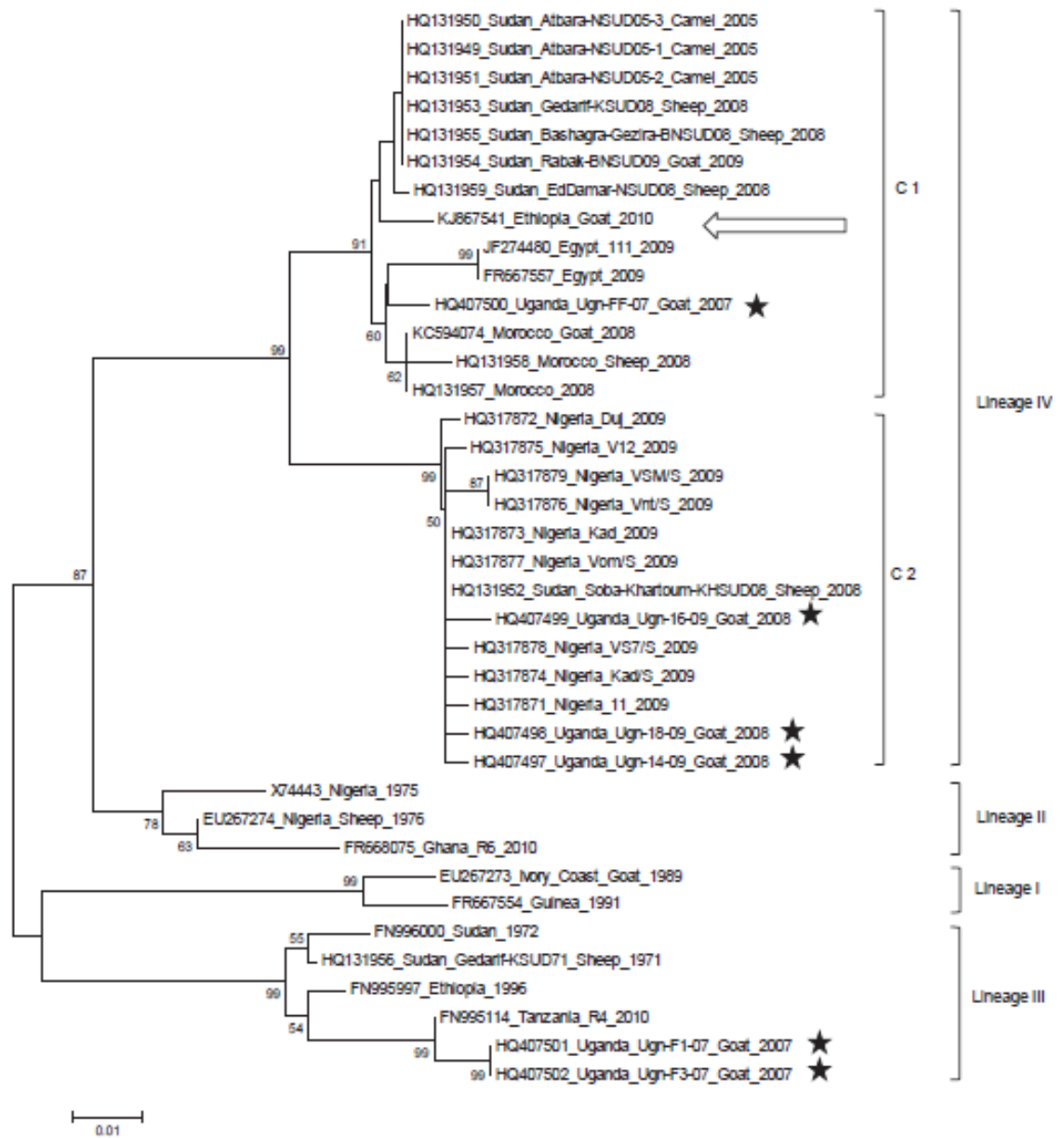
Figure 2.5: Neighbour-joining tree of PPRV-F gene sequences of African PPRV isolates.

The Kimura 2-parameter model with 1000 bootstrap replicates shown next to the branches. The isolate sequenced in this study is indicated by arrow and those from Luka *et al.* (2012) by stars.
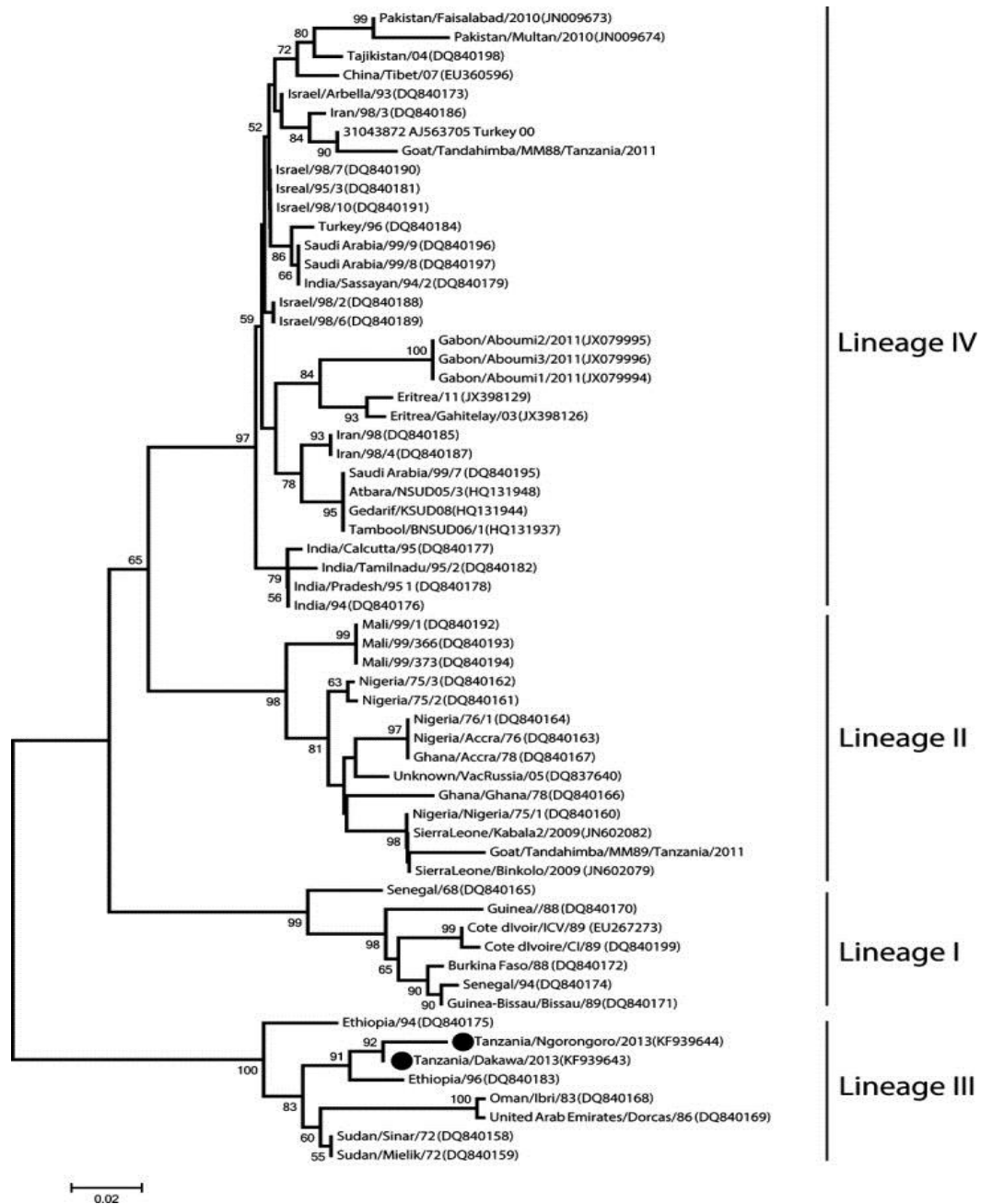
Figure 2.6: Phylogenetic tree of PPRV based on N gene sequences.

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A neighbour-joining phylogenetic tree of Tanzanian PPRV (indicated with circles) (Kgotlele et al., 2014).
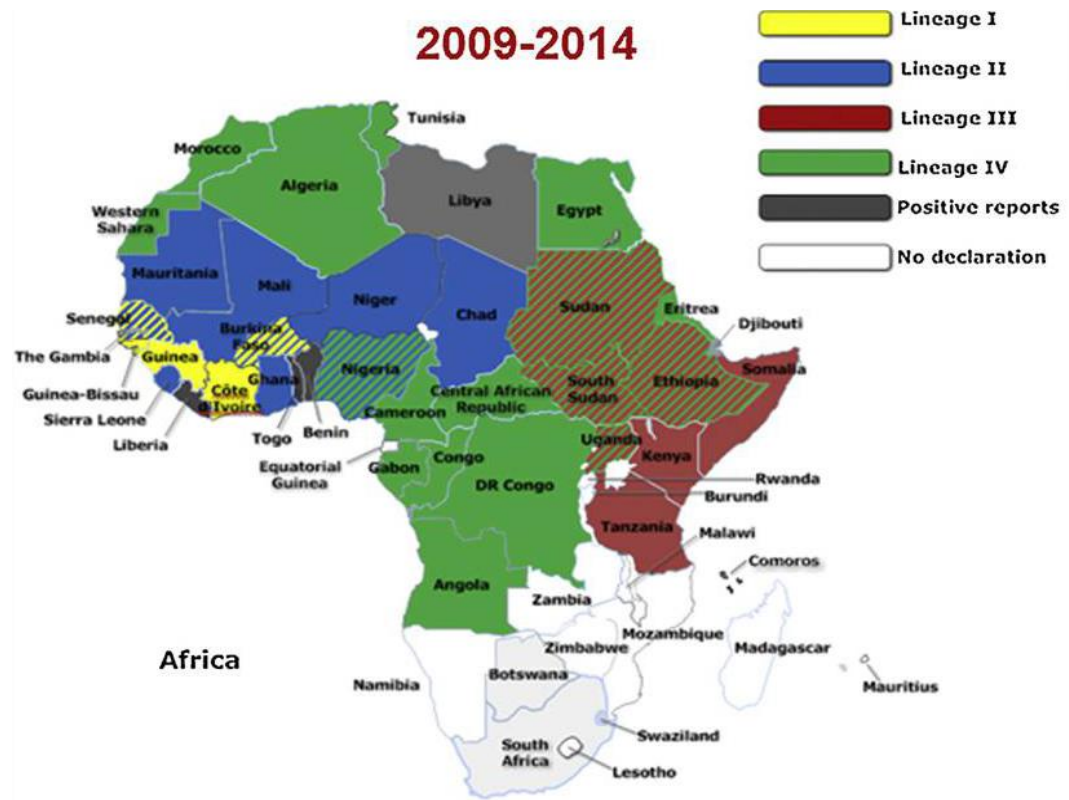
Figure 2.7: PPRV lineage distribution from I-IV (Parida *et al.,* 2016)

2.3.2 Evolution of the disease

Since PPR was reported for the first time in Africa in 1942, the number of involved countries has increased to over 20 between 1988 and 2009 (AU-IBAR, 2013; Parida *et al*., 2016) shown in **Figure 2.8**.

The disease has spread northwards into Morocco (De Nardi *et al*., 2011), Algeria (Kardjard *et al.,* 2015) and Tunisia (Sghaier *et al.,* 2014) and southwards into Tanzania, Zambia and Angola (Banyard *et al.,* 2010; Misinzo *et al.,* 2015; Parida *et al.,* 2015).

Currently, 50 countries are considered at risk for getting PPR while around 70 countries have reported infection to the OIE. Out of these affected countries, more than 60% are in Africa (**Figure 2.9**). Only 48 countries were recognized as PPR free by the OIE in May 2014 (FAO and OIE, 2015).
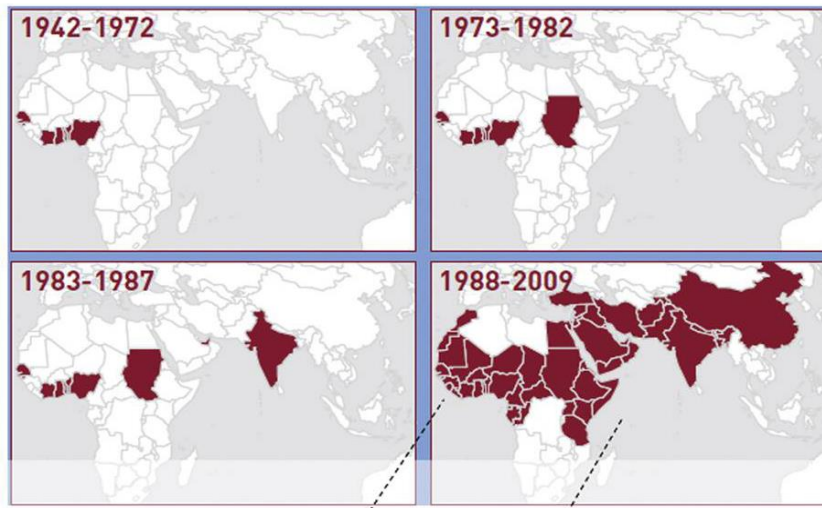
Figure 2.8: Spatio-temporal of PPR (adapted from Parida *et al*., 2015)
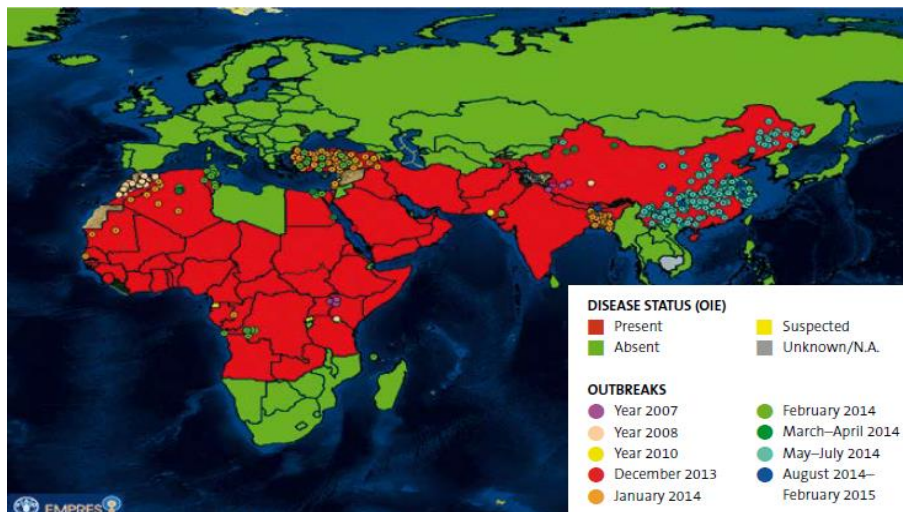
Figure 2.9: Current global PPR situation and occurrence of outbreaks between 2007 and 2015. Source: OIE WAHIS and FAO EMPRES, 2015.

2.4 TRANSMISSION OF THE DISEASE

The virus is transmitted through close contact with an infected animal, although it can also be transmitted through ingestion of contaminated feed and water (Fenner *et al.*, 1993). Transmission is mainly through close contact with secretions/excretions of infected animals such as saliva, faeces, urine, vaginal, oculo-nasal and vaginal fluids and others fluids exchanges or by aerosols holding particles of PPR virus in the expired air (Roeder *et al.,* 1999). Moreover it is important to note that; the survival of PPRV outside of his host is very difficult and limited in a short period of time though the virus is very labile (Gitao *et al*., 2012).

2.5 HOST RANGE

Domestic and wild species including goats, sheep, Impala, Gazelles, Springbuck and camels are potential hosts for PPR disease as described in several studies from Furley *et al.* (1987), Ogunsanmi *et al.* (2003), Sharawi *et al.* (2010) and Kinne *et al.* (2010). In many cases goats are more susceptible compared to sheep and in wild animals gazelles are more affected with the disease compared to other species (Lefèvre and Diallo, 1990). The subclinical form of PPR infection have been reported in cattle, pigs and camels but there is still a need to understand the cross species transmission and specific interactions of strains across species (Taylor, 1984).

2.6 RISK FACTORS ASSOCIATED WITH THE DISEASE

Several reports have shown the host determinant factors of PPR spread, among which age, sex, breed and animal species (Munir *et al*, 2013). From 2008 to 2012, several authors among them Waret-Szkuta *et al.* (2008), Swai *et al.* (2009), Sarker and Islam (2011) and Abdalla *et al*. (2012) found that the sex of the animal was among the determinants for PPR seropositivity risk with female being more affected than male. This is somehow because femals are kept longer than males due to many reasons such as: off-take of male small stock and at an early stage for socio-economic activities and the longer periods for kipping females in herds for productive purposes (Singh *et al.,* 2004b). The stresses associated with milk production and pregnancy stresses may also predispose femals to infection. Sarker *et al.* (2011) found a high PPR seroprevalence in females goats compared to males in Bangladesh. Similar results were observed in Pakistan (Munir *et al*., 2008). Age of animals has been also discovered as an associated PPR risk factor. Luka *et al.* (2011) show that the chances to develop protective PPRV antibody titers in young animals is less compared in old animals and therefore they are more susceptible to PPRV infection. Same results have been found in Kenya, Ethiopia, India, Turkey and Pakistan by Waret-Szkuta *et al.* (2008), Abubakar *et al*. (2009), Singh *et al*. (2004b) and Ozkul *et al*. (2002). The breed was also described as a PPR risk determinants in many herds. In the study done by Lefèvre and Diallo (1990), El Hag and Taylor (1984), Diop *et al.* (2005) and Munir *et al.* (2008) they found that there was high significant differents of PPR distribution among sheep breeds compared to goats

breeds. Thus, there is increased likelihood of sheep breeds (genotypes) having high genetic diversity, with respect to resistance to PPR.

The camel is emerging as a key risk factor in long distance transmission of the disease particularly those used in trade caravans, though PPR has been described in other species of animals (Waret-Szkuta *et al.,* 2008; Swai *et al.,* 2009; Libeau *et al.,* 2011).

Seasonal variation and geography have also been found to be among determinants of PPR spread. Abubakar *et al*., (2009 and 2015) elicited that in rainy season, PPR incidence decreased due to ample amount of fodder availability, leading to increased resistance against the disease. Moreover, large flock sizes, animals visiting animal markets and inadequate veterinary services are risk factors for PPR disease to occur (Zahur *et al.,* 2011). During dry season feed is not available for goats and sheep which result to their closer contact for the few amount of vailable feed disputing that the contact of infected goat and sheep is a route of disesase transmission.

2.7 SYMPTOMS OF THE DISEASE

2.7.1 Clinical signs

The onset of the disease is marked by sudden dullness and fever with high body temperature between 40°C and 41°C (Aiello and Moses, 2011). Other characteristics of the disease include depression, anorexia, severe purulent ocular discharges resulting into matting of the eyelids and blushing of conjunctiva and, severe purulent nasal discharges, respiratory distress and coughing (Diallo *et al.,* 2007). Mucous membrane of the buccal cavity erosions are complemented by marked salivation with ulcers

developing in the mucosa of the alimentary, urinary and respiratory systems (Kumar *et al*., 2004; Chauhan *et al.,* 2009). Most animals develop diarrhoea which may be watery, foul smelling, and/or blood-stained, sometimes containing shreds of tissue (CFSPH, 2008). Diarrhea is accompanied or preceded by a sudden drop in core body temperature followed by death, in fatal cases, five to 12 days after the onset of disease (Baron *et al.,* 2011). Pregnant animals may abort (Abdalla *et al*., 2012) with higher morbidity and mortality rates in young animals than in adults (Baron *et al.,* 2011). Hard non-painful nodules all over the body have also been noted (Gitao *et al*., 2012).

A clear vibrant fluid starts to flow from mouth, eyes and nose, later change the nature and color to become yellow and thick as a result of coinfection with bacteria. Serous nasal and ocular mucopurulent discharges crust over causing sneezing and difficulty in breathing (**Figure 2.10**).Serous to mucopurulent ocular discharges ensue causing matting together of the eyelids (**Figure 2.11**). The epithelial necrosis causes small pin-point greyish areas on the gums, palate, dental pad, lips, inner aspects of the cheeks and upper surface of the tongue has been also described a characteristic symptom to PPR (**Figure 2.12**). Kgotlele *et al*. (2014) found periorbital edema and cutaneous nodules in PPR infected goat in Tanzania (**Figure 2. 13**).

Figure 2.10: Mucopurulent nasal discharge and swollen upper lips (Roeder and Obi, 1999).

Figure 2.11: Mucopurulent ocular discharge matting hair from canthus of the eye (Kihu *et al.*, 2012a).

Figure 2.12: Stomatitis erosion with dead cells on the lower lip of the gums (Berhe, 2006).

Figure 2.13: Periorbital edema and cutaneous nodules (arrows) (Kgotlele *et al.,* **2014).**

2.7.2 Pathology of the disease

The PPR affected animal carcasses are usually emaciated, the hindquarters soiled with soft/watery faeces and the eyeballs sunken. Lips may be swollen and there may be scabs or nodules in late cases. The nasal cavity lining is congested (reddened) with clear or creamy yellow exudates and erosions. The pathology caused by PPR is dominated by necrotizing and ulcerative lesions in the mouth and the gastro-intestinal tract (Roeder *et al.*, 1994). Erosion in the oral cavity is a constant feature affecting the gums, soft and hard palates, tongue and cheeks and into the oesophagus. Abomasum is congested with lining hemorrhages. The rumen reticulum and omasum rarely exhibit lesions. Occasionally, there may be erosions on the pillars of the rumen. The omasum is a common site of regularly outlined erosions often with oozing blood. Lesions in the small intestine are generally moderate, being limited to small streaks of hemorrhages and, occasionally, erosions in the first portions of the duodenum and the terminal ileum. The large intestine is usually more severely affected, with congestion around the ileo-cecal valve, at the ceco-colic junction and in the rectum. In **Figure 2.14** below, the circle shows congested mesenteric lymph nodes while the arrows show congested mediastinal lymph nodes of PPR-suspected animals. Kgotlele *et al.* (2014) found congested lymph nodes in the gastrointestinal and in respiratory system, pneumonia and hemorrhage draining internal organs in a Tanzanian goat suspected with PPR, after postmortem (**Figure 2.14**).
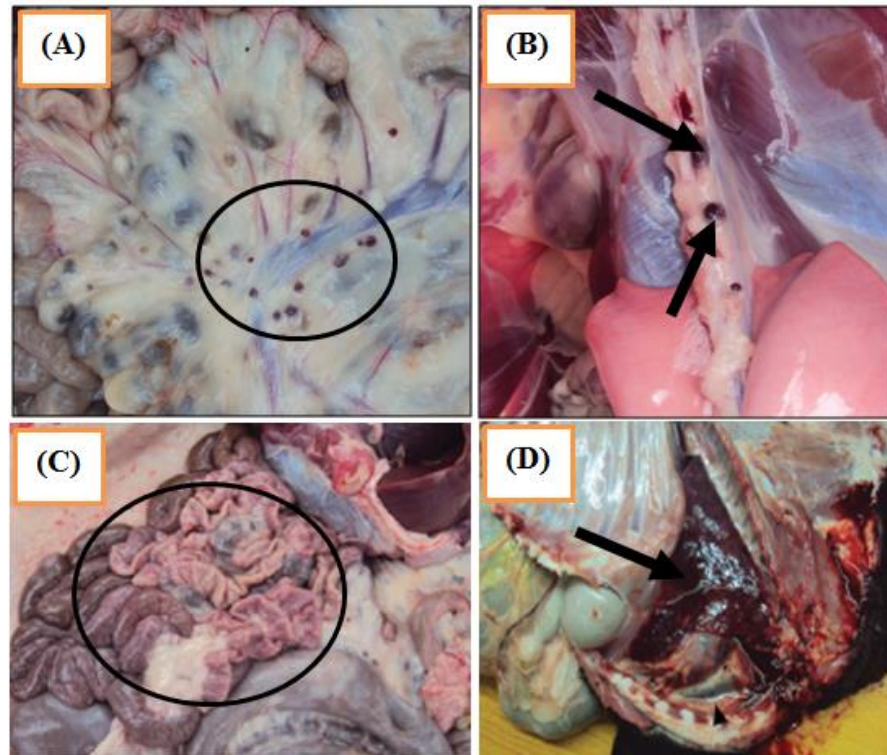
Figure 2.14: Congested lymph nodes in the gastrointestinal tract (A). Congested lymph nodes in respiratory system (B). Congestion of intestines (C). Pneumonia and Hemorrhage draining internal organs (D) (Kgotlele *et al.,* 2014).

Several authors, among them Roeder and Obi (1999) and Troung *et al.* (2014) found in the posterior part of the colon and the rectum, on the crests of the mucosal folds, discontinuous streaks of congestion, referred-to as "zebra stripes". The lung was dark red or purple in color with areas firm to the touch, mainly in the anterior, and cardiac lobes, showing evidence of pneumonia (**Figure 2.15A and B**).
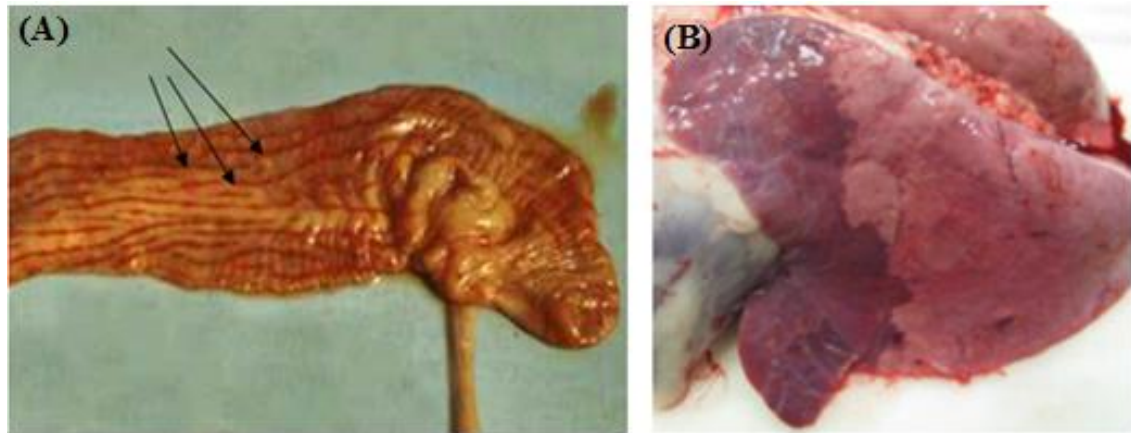
Figure 2.15: PPR in a goat: "zebra striping" in the large intestine (A). Broncho interstitial pneumonia due to PPR infection (B).

2.7.3 Histopathological changes associated with the disease

Peste-des-petits-ruminants virus causes epithelial necrosis of the mucosa of the alimentary and respiratory tracts marked by the eosinophilic intracytoplasmic presence and intranuclear inclusion bodies (Brown *et al*., 1991). Multinucleated giant cells (syncytia) can be observed in all affected epithelia and in the lymph nodes where there is severe depletion of lymphocytes (Troung *et al.,* 2014). Munir *et al.* (2013) showed that, in the lungs, there are multifocal degeneration, ulceration and necrosis, followed by alveolar type II pneumocytes hyperplasia which mostly ends up with syncytial cell formation. Lymphocytes, plasma cells and histiocytes penetration into the alveolar septae leads to its hypertrophy and desquamation with alveolar casts (Munir *et al.,* 2013). Troung *et al.* (2014) found that the intestinal lesions are characterized by blunted villi, degeneration of surface and crypt epithelial cells; lamina propria expansion by a primarily mononuclear infiltration with scattered syncytial cells.

2.8 DIAGNOSIS OF THE DISEASE

Although clinical signs and gross pathological findings may be typical of PPRV infection, they are not exclusive for PPR. This is because respiratory diseases in small ruminants have multiple causes and as a result the disease may go undetected or misdiagnosed as it spreads especially through animals that show no overt clinical signs. *Peste des petits ruminants* (PPR) can be misdiagnosed for capripox (CaP) based on clinical signs and especially during early stage of infection. *Peste des petits ruminants* diagnosis may be performed through isolation of the virus, detection of viral antigens, nucleic acid isolation (viral RNA) and sequencing (Sanger or Next Generation Sequencing); and detection of specific antibody in the serum (Gopilo, 2005).

2.8.1 Virus isolation

This technique involves isolation of the PPR virus in cultured cells (Roeder and Obi, 1999). Samples including: heparinized blood, eye and nasal swabs (from live animals), tonsil, mesenteric lymph nodes, spleen, section of colon and lung from necropsied cases give better chance for virus isolation, since it has been proven that PPR virus circulates in big amount in these liquids (Roeder *et al.,* 1999). Therefore, for successful isolation, samples must be collected during the hyperthermic phase (Lefevre, 1987) and submitted to the testing laboratory in cold ice chain. The most widely used cell culture systems are primary lamb kidney and ovine skin (Gilbert and Monnier, 1962; Laurent, 1968, Taylor and Abegune, 1979; Adombi *et al.,* 2011) and Vero cells (Hamdy *et al*., 1976).

2.8.2 Molecular techniques

2.8.2.1 Nucleic acid recognition methods

The rapid method for molecular diagnosis is the reverse transcription polymerase chain reaction (RT-PCR) technique based on the amplification of whole or any part of the virus genome. However, the N and F protein genes are more appropriate for the specific diagnosis of PPR (Couacy-Hymann *et al.,* 2002; Forsyth & Barrett, 1995). This technique is 1000 times more sensitive than classical virus titration on Vero cells (Couacy-Hymann *et al.,* 2002) with the advantage that results are obtained in 5 hours, including the RNA extraction, instead of 10–12 days for virus isolation. Moreover this technique is very specific because it targets the gene of one's interest in the genome using the specific primers. This technique can be complemented by a more sensitive one, which is real time reverse transcriptase polymerase Chain reaction or quantitative reverse transcriptase polymerase Chain reaction (qRT-PCR) which quantifies the amount of DNA or RNA at each cycle in the thermocycler (Kwiatek *et al.,* 2010; Li *et al.,* 2010). Another molecular technique is the reverse transcription-loop mediated isothermal amplification (RT-LAMP)   which has an intrinsic potential for point of care diagnosis focused on the genetic detection of causative PPR virus (PPRV) in field conditions. This method   uses ammonium sulphate to precipitate the viral envelope and capsid proteins and expose viral RNA, present in the clinical sample, to the LAMP reaction mixture (Couacy-Hymann, 2015; Ashraf *et al.,* 2017). The LAMP based amplification of target nucleic acid is based on isothermal amplification of template DNA utilizing the strand displacement activity of Bst or Bsm DNA polymerase enzyme

originated from *Bacillus stearothermophilus* or *Bacillus smithii*,

respectively (Nagamine *et al.,* 2002). The LAMP products are visualized with naked

eye after the addition of fluorescent DNA-intercalating dyes such as propidium iodide,

SYBR Green I and calcein to the reaction mixture.

2.8.2.2 Specific cDNA Hybridization

This method uses cDNA probes corresponding to the nucleocapsid gene of each virus,

labeled with isotope [P^{32}] nucleotides (Diallo *et al.,* 1989b). This hybridization

technique is used to clearly identify the virus involved in an outbreak (Taylor *et al.,*

1990). Unfortunately, the   technique cannot be used widely because it requires fresh

specimens and the P^{32} isotope has a short half-life;   there are also constraints with the

handling of isotopes due to their reactivity. Diallo *et al.* (1989a) recommended this as a

rapid method for differential diagnosis of infections caused by rinderpest virus (RPV) or

PPRV, since it is derived from the mRNAs for the nucleocapsid protein of each virus,

which can be used to distinguish unequivocally the two virus types rapidly.

2.8.3 Serological tests

2.8.3.1 Viral antigen detecting tests

2.8.3.1.1 Haemagglutination Test (HA)

The haemagglutination test is one of the cheapest, easiest and effective methods for

PPRV diagnosis that has advantage of differentiating PPR from RPV (Johnson and

Ritchie, 1968). Osman *et al.* (2008) used the HA method for detection of PPRV antigen

in forty lymph nodes and spleen samples from suspected cases of PPR in both sheep and goats. The HA test was carried out using goat, chicken and pig RBCs.

2.8.3.1.2 Immunofluorescent Antibody Test (IFAT)

The IFAT is also one of the simple and quick methods for PPRV antigen for diagnosis. It has the advantage that facilities are available in most veterinary laboratories (Last *et al.,* 1994). The IFAT technique is reported to have 100% specificity in detection of PPR antigen in conjunctival smears from suspected PPR cases found from a field outbreak (Sumption *et al.,* 1998). Moreover, the immunofluorescence antibody test (IFAT) is also frequently used as a reference test in validation of new diagnostic methods and estimation of true prevalence (Adel *et al.,* 2016).

2.8.3.1.3 Agar Gel Immunodiffusion Test (AGID)

This method detects virus antigens by the agar gel immunodiffusion method. It is also simple to handle, fast and cheap process. One disadvantage of this test is that it does not discriminate PPR and RP viruses, therefore further tests are needed to do this. Using this test, results can be obtained in one day, but the test is not sensitive enough to detect mild forms of PPR due to the low excreted quantity of viral antigen (OIE, 2013; SADC, 2013).

2.8.3.1.4 Counter immunoelectrophoresis (CIEP)

The method of counter immunoelectrophoresis works almost in the same principle as the AGID except that the gel has an electric charge to improve the test sensitivity. Counter immunoelectrophoresis is one of the most rapid tests for detecting viral antigen. It has been shown that both CIEP and AGID are group-specific and can't differentiate PPR and RP infections (Obi and Patrick, 1984). Balamurugan *et al.* (2014) also showed that the CIEP was comparatively more sensitive and rapid method than that of AGPT, but could not differentiate between RPV and PPRV infection.

2.8.3.1.5 Immunoperoxidase Staining (IP)/Immunohistochemistry (IHC)

This method involves the combination of histopathology with immuno histochemical staining (immuno peroxidase). It is a useful test performed on formalin–fixed material and can discriminate between RP and PPR when performed with specific monoclonal antibodies. The IHC reaction is characterized by the presence of light to dark brown, fine to coarse granular areas in cells and tissues (Kumar *et al.,* 2004). Chen *et al.,* (2010) confirmed that immunohistochemistry (IHC) is one of the useful research tools used to localize specific antigens in tissue sections with labeled antibodies based on antigen-antibody interactions. The marker including fluorescent dye, enzyme in general, radioactive element or colloidal gold can be used for immune reactive products visualization.

2.8.3.1.6 Immunocapture ELISA (IC-ELISA)

The immunocapture ELISA (ICE) method can detect rapidly and sensitively the viral antigens. Moreover, this test can differentiate between PPR and rinderpest. Considering the similarity in geographic distribution of the two diseases and knowing that they can affect the same animal, this method seems to be of a great importance (Diallo, 2000; Diallo, 2004). Although an old method, the immunocapture ELISA (IcELISA) method for PPRV antigen detection was still used recently to detect PPRV and respiratory viruses' antigen in dromedary camels in Sudan (Saeed *et al.,* 2015).

2.8.3.1.7 Sandwich ELISA (S-ELISA)

Peste-des-petits-ruminants virus-specific neutralizing monoclonal antibodies are used in a simple and rapid double-antibody Sandwich ELISA for specific detection of PPRV antigen in goat/sheep tissues and secretions (Saliki *et al.,* 1994). Singh *at al.,* (2004a) described a sandwich ELISA test using PPR specific monoclonal antibodies (clone 4G6) to an epitope of nucleocapsid protein. This technique is also known to be simple, rapid, cost effective and convenient for intensive clinical surveillance and routine diagnosis of the disease (Singh *et al.,* 2004a). The results from a study on comparative evaluation of RT-PCR with sandwich-ELISA for detection of *Peste des petits ruminants* in sheep and goats in India showed a low sensitivity and specificity by F-gene based RT-PCR when compared with sandwich ELISA suggesting that some other highly sensitive and specific primers should be explored for detection of PPR by RT-PCR (Mahajan *et al.,* 2013).

2.8.3.2 Antibody detecting tests

2.8.3.2.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Laboratory confirmation is needed to detect PPR amid a number of other acute diseases with grossly similar presenting signs. Conventional serological tests such as haemagglutination test, enzyme linked immunosorbent assay (ELISA) and virus neutralization assay (Ularamu *et al*., 2012) often fail to differentiate PPR from rinderpest (Jalees *et al.,* 2013). An alternative system for sero-surveillance and sero-monitoring of PPR is a monoclonal antibody based competitive-ELISA, (cELISA) (Singh *et al*., 2004a). The cELISA test is based on the competition between the anti-PPR monoclonal antibody which is directed against the haemagglutinin protein of PPR virus and the antibodies in the serum sample (Anderson *et al.,* 1991). Competitive Enzyme-Linked Immunosorbent Assay sensitivity is 99.4 % and specificity is 94.5%. The presence of antibodies to PPR virus in the serum samples blocks reactivity of the monoclonal antibodies which causes reduction in the expected color following the addition of enzyme labeled anti-mouse conjugate and chromogen solution (Khan *et al*., 2007). The test has high degree of sensitivity (92.2%) and specificity (98.4%) for disease serological surveillance and is very convenient for large sampling frames (Singh *et al*., 2004a and b). The blocking ELISA (B-ELISA) is proved to be sensitive, specific, simple and more rapid for detection of PPR antibodies (Saliki *et al.,* 1993). Unfortunately the VNT, B-ELISA may be less affected by the contamination and the quality of sera such as cytotoxicity (Saliki *et al.,* 1993).

2.8.3.2.2 Haemagglutination Inhibition Test (HI)

This technique endeavors to absorb out the cross reacting antibodies to rinderpest antigen from a PPR serum and leaving the specific antibody to PPR which is determined by haemagglutination-inhibition test (Wosu, 1985). The HI test is cheaper, simple and reliable. However, there is a need to standardize the commonly available PPR antigen for easy evaluation of efficacy of PPR vaccination efforts.

2.8.3.2.3 Counter Immunoelectrophoresis (CIEP)

The CIEP is one of the highly adaptable methods for serum antibody titration but can also be used for sero-epidemiological and experimental surveys to diagnose PPR (Majiyagbe *et al.,* 1984). It was previously confirmed that its rapidity, simplicity and sensitivity made it a suitable technique in serological studies of PPR (Durojaiye and Taylor 1984).

2.8.3.2.4 Agar Gel Diffusion (AGID)

The test is used to detect antibodies against PPR in the sera of the affected goats or sheep (Durojaiye, 1982). This method involves passive diffusion of soluble antigens and/or antibodies toward each other leading to their precipitation in a gel matrix. It is also called ouchterlony test, double immunodiffusion test or agar gel precipitin (AGP). This test is considered to be useful for diagnosis of PPR in the field.

2.8.3.2.5 Virus Neutralisation Test (VNT)

The virus neutralization test (VNT) is sensitive and specific, but more expensive and time-consuming. This test is the oldest most reliable test for detection of morbillivirus antibodies (Rossitter et al., 1985). Serum against either PPR or RP may neutralize both viruses, but would neutralize the heterologous virus at a lower titer compared to the homologous virus. Therefore, for differentiation purposes, reciprocal cross neutralization is used (Taylor and Abegunde, 1979). Hu et al. (2012) in a study on rescue of recombinant peste des petits ruminants virus: creation of a GFP-expressing virus and application in rapid virus neutralization test, found that recombinant virus allowed more rapid and higher throughput assessment of PPRV neutralization antibody titer via the virus neutralization test (VNT) compared with the traditional method.

2.8.3.2.6 Precipitinogen Inhibition Test (PIT)

The success of PIT test which is one of the oldest serological tests is basically based on the ability of antibody in serum to inhibit diffusible virus antigen (precipitinogen) from developing a precipitin line against hyper immune serum in AGPT. Durojaiye (1987) observed that the PIT test is more sensitive (33%) as compared to neutralization test (NT) (28%).

2.9 VACCINE AND VACCINATION OF THE DISEASE

In the past, vaccination with the rinderpest vaccine was used to control PPR because of the existence of a strong antigenic relationship between PPR and rinderpest viruses (OIE, 2008). Currently PPRV homologous vaccine made from strain Nigeria PPRV

75/1 LK6 Vero 70 is in use in control of PPR in endemic areas. New *PPR* recombinant marker vaccines are under development, they will enable differentiation between infected and vaccinated animals for sero-surveillance and sero-monitoring purposes (Diallo, 2006). Strong support of diagnostics and timely vaccination of the susceptible population based on an understanding of the epidemiology of the disease may help eradicate PPR as it was done with rinderpest (Balamurugan *et al.,* 2011). Different types of PPR vaccine including conventional, thermostable, recombinant and edible vaccines have been developed and used for control/eradication of the disease worldwide (Abubakar *et al.,* 2012; 2015). The vaccine lineages should match with field isolates lineages for a better control of the disease. Genetic characterization of field PPRV strains provided foundations for construction of vaccines from domestic strains as has recently been practiced in India (Anees *et al.,* 2013). The rinderpest vaccine was used up to the 1990s but was replaced by the live-attenuated PPRV vaccine developed from the Nigeria 75/1 strain (Luka *et al*., 2011) following the start of the Global Rinderpest Eradication Programme. A differentiation of infected from vaccinated animals (DIVA) would improve epidemiological data by allowing tracking of infection in areas where there has been partial vaccination. Efforts are being made to develop thermoresistant vaccine and PPR recombinant marker vaccines (Berhe, 2006; Diallo, 2006). The recombinant marker vaccines will make it possible to differentiate infected and vaccinated animals for sero-surveillance and sero-monitoring purposes while thermoresistant vaccine will reduce the cost of vaccination by side-stepping the cold chain storage.

Holzer *et al.* (2016) underlined the utility of these constructs as DIVA vaccines for use in PPR control based on recombinant viruses showing how in a local breed of goat in a country, where PPR disease is common, such as Kenya, as little as 10^7 pfu of adenovirus expressing the PPRV-H glycoprotein(AdH) gave significant protection against PPRV challenge, while a vaccine consisting of 10^8 pfu of each of AdH and adenovirus expressing the PPRV F glycoprotein (AdF) gave apparently sterile protection.

2.10 CONTROL AND PREVENTION OF PPR

Different control and preventive strategies can be used for surveillance or eradication of PPR in animals. Separation of infected animals from healthy animals at the first stage can minimize the chance of transmission of the virus from infected to healthy animals. At the second stage, slaughter of apparent disease and sero-positive animals, coupled with proper disposal of all infected material and decontamination of items of infected sheep/goat flock is crucial for control/ eradication of PPR as suggested by Diallo *et al.* (1989 a and b) and Worrwall *et al.* (2009). However, vaccination of animals seems to be a good option to minimize the risk of occurrence in any healthy animal flock. Worldwide, different immunization strategies against PPR have been used such as attenuated tissue culture rinderpest vaccine (TCRV), immunization of small ruminants with lymph node and spleen materials containing virulent virus inactivated with 1.5-5 % chloroform, have been used; while, currently, PPR homologous vaccine is available which is prepared by a new freeze-drying process and addition of stabilizing agents. Proper disposal of carcass and contact formites, decontamination and restriction on

importation of sheep and goats from affected areas can help control PPR (Chauhan *et al*., 2009).

2.11 GLOBAL ERADICATION STRATEGY OF PPR

Bearing in mind the strong negative impact that PPR can have on food security and the livelihoods of poor main keepers of sheep and goat farmers in Africa and Asia, the Global Framework for the Progressive Control of Transboundary Animal Diseases (GF-TADs), Global Steering Committee in 2012, the Food and Agriculture Organization of the United Nations' (FAO) Council and the Committee on Agriculture (COAG) and the World Organisation for Animal Health (OIE), in the form of a resolution of the world assembly of delegates of the OIE in 2014, have all recommended the development of a PPR Global Control and Eradication Strategy (hereinafter named 'Global Strategy') by 2030 and expressed a strong willingness to address the animal health problems in a systematic way, dealing with horizontal as well as vertical issues (FAO, 2015; OIE, 2015). The plan considered the PPR current status, the requirement control tools at regional and global levels and effective cost (between USD 7.6 and 9.1 billion), timeline (15years), and key stakeholders; considering that PPR Strategy cannot be a 'stand-alone' activity.

2.12 SEASONALITY AND SOCIAL ECOLOGY OF PPR

The increase of PPR outbreak incidences has been attributed more to an increased number of susceptible animals compared to seasonal upsurges according to Taylor *et al.* (1990). Moreover, in their previous studies Abubakar *et al.* (2009) and Munir *et al.*

(2008) mentioned that lack of water due to long dry spells or winter and pastures results in poor livestock nutrition; consequently small ruminants become weak and dilapidated with lowered immunity against PPR. Therefore, the increased animal movements in search of better nutrition and shelter against the adverse climatic conditions contribute significantly to the spread of the PPR to susceptible groups (Singh *et al.,* 2004b).

Gopilo (2005) found that the seasonal epidemiologic patterns of the PPR disease differ in different geographical areas and ecological systems. In Pakistan for example, seasonal PPR outbreaks were observed by Abubakar *et al.* (2009 and 2011); Munir *et al. (*2008); Balamurugan *et al.* (2012a and b) and Singh *et al.(*2004b), suggesting that seasonal grazing patterns among nomadic livestock keepers during winter encourage transmission of diseases . Similar observations were made by Sarker *et al*. (2011) who associated PPR outbreaks in Bangladesh to winter grazing.

Some results showed that PPR outbreaks have been attributed to the cessation of rinderpest vaccination and loss of antibody cross protection between the RP and PPR, leaving the small ruminants fully exposed to PPRV and the fact that spread of the disease outbreaks has for a long time been associated with cultural and socio-economic activities such as livestock trade, disasters, cultural festivals, change of husbandry practices, conflicts, nomadism and seasonal climatic and environmental changes (Libeau *et al.,* 2011). Seasonality of PPR in Ethiopia has been attributed to seasonal animal movement in search for pasture and water during dry seasons but also the social exchange of animals and livestock marketing which exhibit seasonal patterns with peak

outbreaks being experienced in March-June and October-November (Gopilo, 2005; Waret-Szkuta *et al.,* 2008).

2.13 SOCIO-ECONOMIC IMPACTS OF THE DISEASE

Small ruminant, especially sheep and goats are considered as "mobile banks" and referred to as "poor man's cow". These animals are mainly kept to generate income through milk and meat productions (economic role). Moreover, goats and sheep have a cultural importance because they are more used during religious and traditional events (FAO, 2009a). In D. R. Congo and many other African countries, goats and sheep are also more readily marketed than large ruminants and are often slaughtered for home consumption, as large ruminants are too much for a family; most of it will remain and get spoilt; these large animals like cattle are normally utilized in big celebrations and for dowry. Elsawalhy *et al.* (2010) demonstrated that goats and sheep provide a high social status to individuals and household and also serve as the much envied symbol of wealth and respect amongst pastoral communities. Therefore, by inflicting high losses to the small livestock due to high mortality and morbidity rate, PPR is classified among the major threat for income generation and impacts on the livelihoods and food security of the poor and marginalized segments of society as reported by the World Animal Health Organization (Khalafalla *et al.,* 2010). Perry *et al*., (2002) ranked PPR in the top ten diseases of small ruminants. The disease has also been ranked by pastoral communities as one of the top ten diseases of small ruminants (Diallo, 2006). According to the Food and Agriculture Organization, around 62.5 % of global goat and sheep's population are at risk of getting PPR infection (FAO, 2009b). Moreover, there

are indirect costs due to treatment, loss of animal body condition, reduction in market value, increased cost for veterinary services and labour due to PPR. The report from the PPR Global Control and Eradication Strategy meeting for PPR eradication showed that *PPR* has infected animals in around 70 countries in Middle East parts of Asia and Africa. The disease causes per losses around 1.5 to 2 billion USD in countries that have 330 million of poorest people many of whom depend on them for their livelihoods and own 80% of total goats and sheep found in the world (FAO, 2015; OIE,2015). Moreover, it was estimated in the same report a total of 2 and 1.7 billion USD of direct losses each year due to PPR infection. The PPR vaccination costs seems to be low ranges between USD 270 and 380 million annually compared to the impact of PPR alone valued at between 1.45 and 2.1 billion USD each year (FAO, 2015; OIE, 2015).

In India, Chauhan *et al.* (2009) reported an annual loss of 39 million USD due to PPR infection, 1.5 million USD in Nigeria (Hamdy *et al.,* 1976). Few years later, Stem (1993) indicated that vaccination against PPR in Niger was highly valuable, with an anticipated gain of 24 million USD in five years by investing two million USD.The Southern African Development Community (SADC) report of 2012, confirms that since its emergence in 2010 to June 2012 in D.R. Congo, 120,000 small ruminants have died in PPR outbreaks with an estimation of 600 000 sheep and one million goats being considered to be at risk of getting infection (SADC, 2012a). These animals are representing one-quarter of goats and two-thirds of sheep in the whole country. The annual direct loss due to *PPR*, i.e. costs of died sheep and goats evaluated at 5.3 million USD (SADC, 2012b).

2.14 MIXED INFECTION OF PPR AND OTHER DISEASES

Concurrent or mixed infection with two or more pathogens is a common occurrence (Cho *et al.,* 2006; Saravanan *et al.,* 2007; Ozmen e*t al.,* 2009; Mondal *et al.,* 2009; Behera *et al.,* 2010). The knowledge about the interaction between several pathogens when they occur in concurrent infections is still limited (Malik *et al.,* 2011). Although goatpox (GP) is not associated with very high mortality in some African countries, its occurrence reduces the profitability and income of the farmers. *Peste-des-petit-ruminants* has been classified as an immunosuppressive disease with co-infection that can increase the incidence and severity of associated diseases in small ruminant population (Rajak *et al.,* 2005). Malik *et al*. (2011) found that PPR was more common in the Indian native goats than BT or GP, but the heavy mortality observed in goats could be attributed to the mixed dual or triple infection of PPR, pox and BT. Mondal *et al.* (2009) confirmed the same in small ruminants. The role of PPRV for suppressing the immune system of the goats should not be ignored in the case that the surge of a particular virus antigen may be due to acute infection or flare up of virus that was already present in the animals because of stress or immunosuppressive conditions. Moreover, few cases of mixed viral infections in sheep and goats involving PPRV, orf virus, goat pox virus or BTV have been reported in India and other countries (Saravanan *et al*., 2007). Recently, Karim *et al.* (2016) confirmed the outbreak of disease in goats with high mortality rate due to mixed infection of PPR and goatpox detected for the first time in North-East India. The occurrence of dual infection of PPR and goatpox in indigenous goats was earlier reported in China and required appropriate

control measures since goatpox virus can be a threat and can exhibit change in host specificity and pathogenesis of PPR (Yan *et al.,* 2012). The problem doesn't seem to be the two diseases looking alike (misdiagnosis) but that when they occur together their tends to be enhancing effect of the respective diseases caused; that is: there is potentiation.

2.15 GOAT GENETIC DIVERSITY AND SUSCEPTIBILITY TO PPR

Indigenous goats are adapted for resiliency and sustainability in diverse African ecosystems with untapped potential for production improvement (Naderi *et al.,* 2012; Tarekegn *et al.,* 2016). Single nucleotide polymorphism (SNP) based approaches provide opportunities to improve on the limitations faced by traditional quantitative studies to accelerate genetic progress (Lashmar *et al.,* 2015). The D.R. Congo has a big number of goat populations; unfortunately, these goats are not characterized for a better management and selection in the integrated breeding programs. There is need to characterize the goat's gene pool, with respect to *Peste des petits ruminants* outbreaks to reveal the possible origin and routes of introduction into the country, which could explain the spread of the disease and the occurrence of genes that are susceptible to the disease. Six goats lineages (A-B{B1,B2}-C-D, F and G) were found with molecular studies on mitochondrial DNA (mtDNA *d-loop*) region sequencing (Luikart *et al.,* 2001; Naderi *et al.,* 2007) (**Table 2.1**). Unfortunately, previous studies on mitogenomes proved the complexity in the process of the domestication of goats (Nomura *et al.,* 2013) and (Doro *et al.*, 2014). Pereira *et al.* (2005) found that, lineage A was found to be more distributed geographically.

Moreover, Zeder and Hesse (2000), Naderi *et al.* (2008) concluded that most likely the Eastern Anatolia to be the origin of this lineage, where it is common in wild populations. One the other hand haplogroup B was shown to have originated from southern and eastern Asia. Haplogroup C is found in Switzerland, the Slovenia republic, India and Pakistan, while D is very rare and was found exclusively in native goats from Indian and Pakistan. Haplogroup F is found only to Sicily and G in Turkey, Saudi Arabia, Iran, Kenya and Egypt as described by Naderi *et al.* (2007), Kibegwa *et al.,* (2015).

Table 2.1: The six haplogroups/ lineages of goats with reference numbers in the gene bank (NCBI).

Haplogroups	Accession numbers	Reference
A	AY155721	Joshi *et al.,* 2004
A	EF618134	Naderi *et al.,* 2007
A	EF617779	Naderi *et al.*, 2007
A	EF618200	Naderi *et al.*, 2007
A	EF617945	Naderi *et al.*, 2007
A	EF617965	Naderi *et al.*, 2007
B1	AB044303	Mannen *et al.,* 2001
B1	EF617706	Naderi *et al.,* 2007
B2	AJ317833	Luikart *et al.,* 2001
B2	DQ121578	Liu *et al.,* 2006
C	AY155708	Joshi *et al.,* 2004
C	AJ317838	Luikart *et al.,* 2001
C	EF618413	Naderi *et al.,* 2007
C	DQ188892	Liu *et al.,* 2005
D	AY155952	Joshi *et al.,* 2004
D	EF617701	Naderi *et al.*, 2007
D	DQ188893	Liu *et al.,* 2005
F	DQ241349	Sardina *et al.*, 2006
F	DQ241351	Sardina *et al.,* 2006
G	EF618084	Naderi *et al.,* 2007
G	EF618535	Naderi *et al.,* 2007
G	EF617727	Naderi *et al.,* 2007

CHAPTER THREE: SEROPREVALENCE, DISTRIBUTION AND RISK FACTORS OF *PESTE DES PETITS RUMINANTS* IN SOUTH KIVU.

3.1 INTRODUCTION

The small ruminants, especially goats and sheep are a very essential livelihood asset for the South Kivu pastoral community of Democratic Republic of the Congo. *Peste-des-petits-ruminants*, a transboundary small ruminants viral disease that largely affect goats, sheep and wild species (springbuck, gazelles and impala) as well as in camels as described previously by Banyard *et al*. (2010), Sarker and Islam (2011) and Muse *et al.* (2012b). Moreover, several studies have shown that in Sub-Saharan Africa, the Middle East and Asia, PPR virus is endemic (Taylor, 1979; Ismail and House, 1990; Housawi *et al.,* 2004; Swa *et al*., 2009; Munir *et al*., 2013). Muse *et al.* (2012) showed that PPR virus is transmitted through close or direct contact between infected and non-infected animals but also through exchanges of fluids by oral, nasal, ocular or respiratory routes. Clinically, PPR is characterized by muzzle and lips proliferative and maculopapular rash of infected animals followed by serous nasal and ocular discharge which becomes mucopurulent, high fever, weight loss, and secondary infections resulting from the immunosuppression caused by the virus (Kayunze *et al.,* 2012). Trans border movements of animals, farming systems, trade and other socio-economic factors could be the main predictor for introduction, transmission and maintenance of PPRV in some areas that were still considered to be PPRV free (Kaukarbayevich, 2009; Zhao *et al*., 2010). Described in 1942 for the first time in Ivory Coast in West Africa, the disease

has spread into several countries of the sub-Saharan Africa (SADC, 2012b; FAO, 2016). In D.R Congo the disease was reported since 2008 causing high economic losses to farmers (SADC, 2012c). Previous studies from Dhar *et al.* (2002), Ozkul *et al.,* (2002) and Kwiatek *et al.* (2007) demonstrated that small ruminants including goats and sheep can develop positive level of antibody titer against PPRV under natural situation.

Cattle are able to seroconvert in case of a high prevalence of PPRV in small ruminants, although they are not susceptible to the disease and only develop abortive infections unable to transmit to naïve animals (Abubakar *et al*., 2011). The objective of this study was to determine the PPRV antibodies prevalence and associated risk factors of PPR in South Kivu with a view of identifying the high risk areas and strengthening control strategies for the disease.

3.2 MATERIALS AND METHODS

3.2.1 Study area

The current study was carried out in South Kivu province, located in Eastern of D. R. Congo, which is found in central Africa (**Figure 3.1**) where PPR outbreaks have been reported, based on clinical manifestations, with a huge loss impact since 2008 to date. South Kivu region shares borders with North Kivu to the North, Kivu Lake to the North East, Maniema to the West, Katanga to the South and Burundi, Rwanda and Tanzania countries to the East (**Figure 3.2**). It is located at 3.0167° S, 28.2667° E. The size area is about 65 070 km^2, with a total population size of 4 614 768 (71persons per km^2). The Koppen-Geiger Climate classification system classifies its climate as tropical wet and

dries (Aw) and the altitude is 1531 meters above the sea level; has an average rainfall of about 1 500 mm, and more than 50 % of the total land used for grazing. This study was conducted in 24 randomly selected villages and equaly selected within four territories (Mwenga, Shabunda, Fizi, and Kalehe) out of the seven of South-Kivu and where PPR outbreaks have been reported sporadically (FAO, 2012a, IPAPEL, 2016).
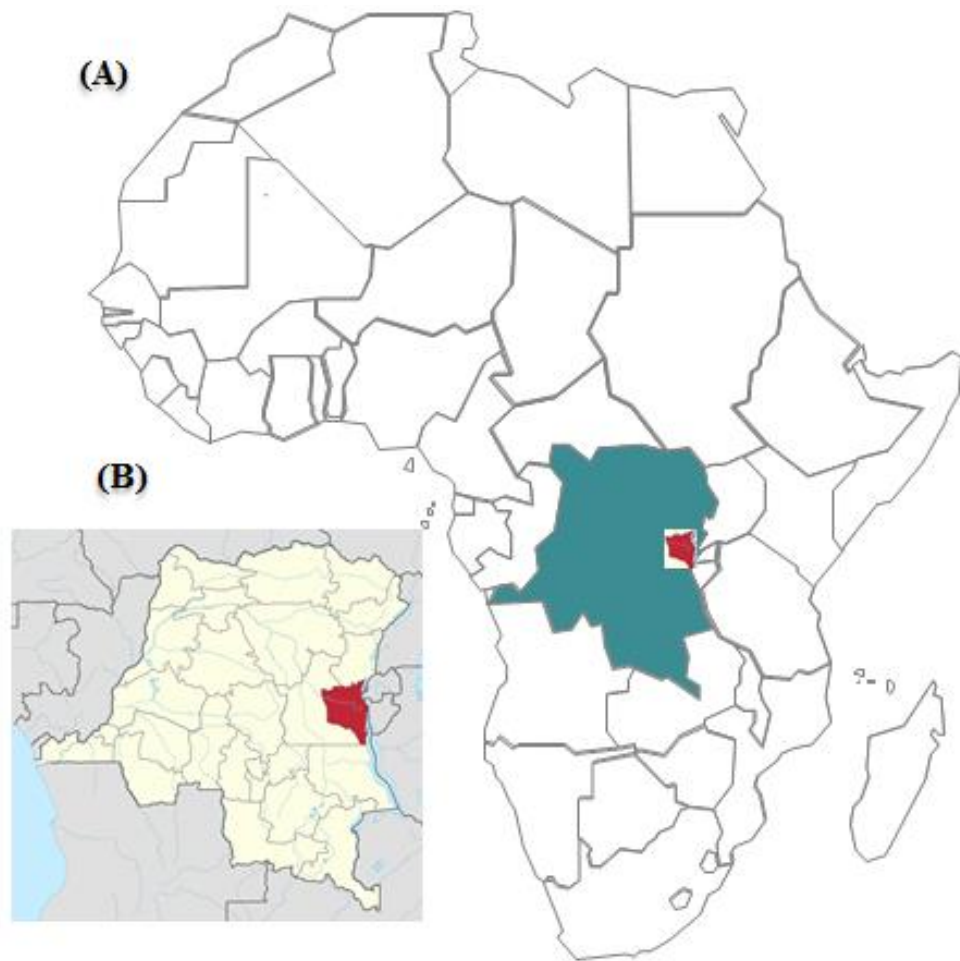
Figure 3.1: Map of Africa showing D.R. Congo in dark green and South Kivu province in red (GIS lab-UEA, 2015).
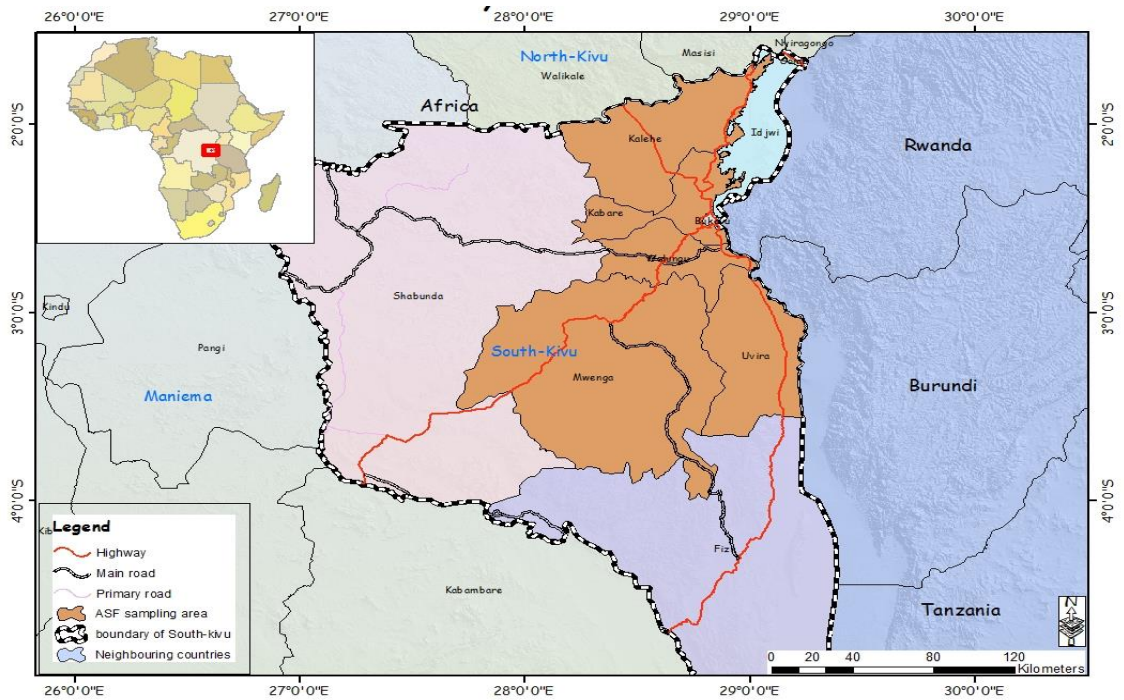
Figure 3.2: Map of South Kivu with administrative divisions showing the sampling area (FAO, 2012; Central Intellence Agency, 2014).

3.2.2 Study design and sampling points

The study was cross-sectional conducted in four different agro-ecological regions in South Kivu at various points (**Figure 3.3**) located in Eastern part of D.R. Congo. An overall of 320 serum samples from non-vaccinated asymptomatic sheep and goats were picked randomly from targeted regions. The age groups of sampled animals were: less than 5 months (to rule out maternal antibody); between 5-12 months and over 12 months (to identify recent infection). Counties and villages were selected purposively in clusters based on PPR outbreak history report; this was followed by a random selection of animals within the selected villages. For serum collection, a sample size was

47

estimated based on priori prevalence from the Rapid Epidemiological Assessment study

described earlier p =22.1% (Diallo *et al.,* 2007), using the formula:

$$n = z_{\alpha}^{2} \times [p \times (1\text{-}p)/L^{2}]$$

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

Where; z_{α} = 1.96, α = 0.05, p = 0.221, L = 0.05; L^2 is the standard error or the desired

level of precision or accuracy, n is the sample size, p the prevalence, α or z is a

confidence level at 95%, D the design effect and N the corrected sample size. An

average number of animals per village, m=6 with a Design effect, D=0.936.

The sample size was now corrected to be 320 serum samples for serological analysis by

the design effect value using the formula:

$$n_{new} = N = n \times D;\ \text{with a precision of 95\% confidence.}$$

A larger number of goats were considered due to their higher susceptibility to the

disease compared to sheep and the huge number registered in D. R. Congo compared to

sheep (Lefèvre and Diallo, 1990; Dhar *et al.,* 2002, IPAPEL, 2016).
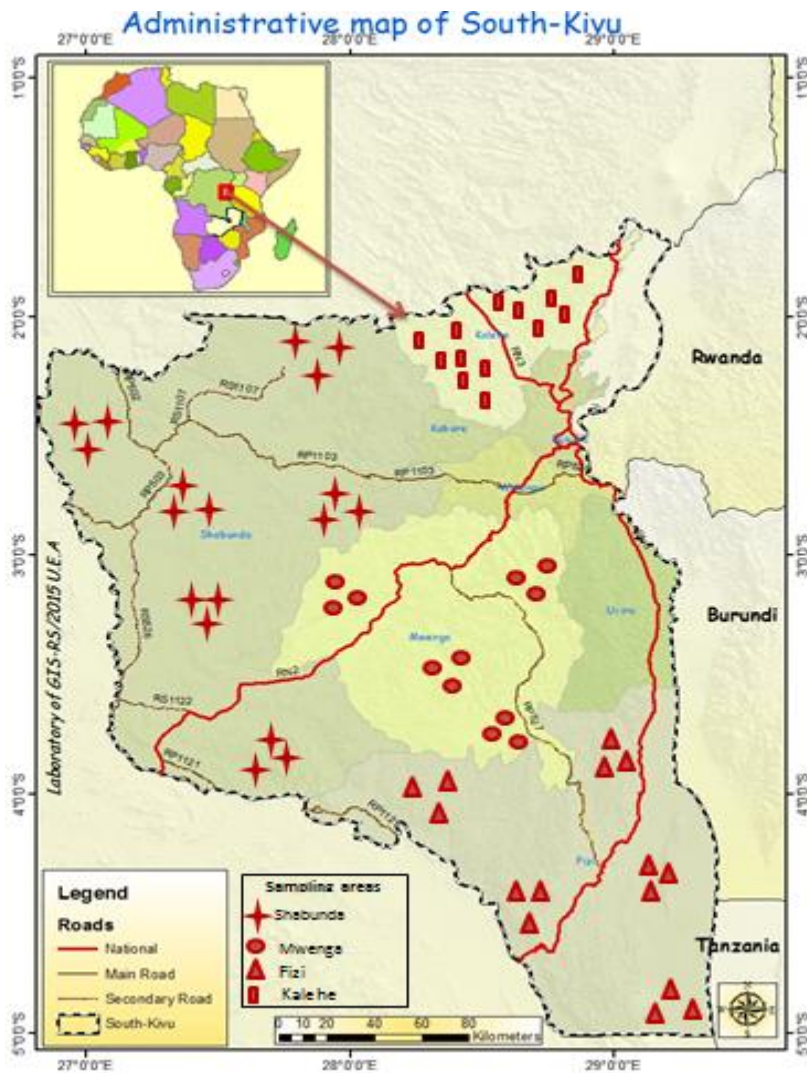
Figure 3.3: Map of animal sampling regions in South Kivu (GIS lab UEA, 2014).

3.2.3 Sample collection and preparation

Whole blood was collected by jugular vein puncture, placing the blood into sterile vacutainer tubes of 5 ml (BD Biosciences, Franklin Lakes, USA) and left to clot overnight at room temperature for serum extraction. Serum was decanted into sterile

cryovials and kept on ice during transportation to the laboratory. At the International Livestock Research Institute-ILRI Hub laboratory, the serum samples were stored at -80° C. Each tube was labelled using codes referring to the village, district and the number of the questionnaire where several parameters for risk factors information were collected.

3.2.4 Sample analysis

To assess the existence of the anti-PPRV nucleoprotein (N) antibodies with competitive enzyme linked immunosorbent assay (cELISA) using Innovative diagnostics kit (ID vet, France) from France (ID Screen$^®$ PPR competition, www.id-vet.com) following the manufacturer's instructions (**Appendix 1**), the serum samples were analyzed. Briefly, the polystyrene plates coated with PPRV antigen were used. To each well, serum and monoclonal antibody (mAb) against the PPRV-N protein were added, followed by the secondary antibody labeled with the HRP enzyme. Substrate was then added and the plate read at 450 nm using ELISA DATA Interchange (EDI) software to give optical density (OD) values. Percentage of competition (S/N) values were calculated using the following formula:

$$\text{S/N} = \left(\frac{OD \ of \ each \ duplicate \ sample}{OD \ Negative \ control}\right) \times 100$$

Samples with S/N value of ≤50% were considered positive for PPRV antibodies, sample with 50<S/N%≤60% were considered to be doubtful, while samples showing S/N value >60% were considered to be negative. *Peste des petits ruminants* seroprevalence map was generated using ArcGIS. The seroprevalence was calculated

using the proportion formula. Statistical analysis was done using Sigma Plot 13.0 for histograms, JMP-Pro software and Stata 11 for uni and multiple variate analysis.

3.2.5 Determinants factors and economic impact of PPR

Laboratory results were complemented with data on the respective 20 factors collected using structured questionnaire for respective farmer (**Appendix 2**) to identify risk factors associated with PPR prevalence and seropositivity. The significant associated factors found from univariate analysis using chi-square, were further analyzed multivariable by logistic regression.

3.2.6 Data Analysis

Epi Info™ and Microsoft Office Excel 2013 were used to calculate frequencies of PPRV samples prevalence. Chi-Square (X^2) test was used to test the significance of proportions between animals which tested negative and those which tested positive. F-test for multiple logistic regressions at 95% confidence interval (CI) was used to determine the correlation between the serological status of the animals with the associated PPR determinants. The antibody distribution maps of PPR were then generated using ArcMap 10.3 software.

3.3 RESULTS

3.3.1 Prevalence of PPRV antibodies in the unvaccinated goats and sheep

The overall PPRV specific antibodies prevalence was 45.3% (145/320), with higher prevalence in goats (53.3%; 128/240) compared to sheep (21.3%; 17/80). Shabunda and Mwenga regions had the highest PPR antibody prevalence at 65.2 and 64.1%,

respectively, in goats and 26.3 and 18.1 %, respectively, in sheep, followed by Fizi then

Kalehe regions, with 48.2 and 20% respectively in goats, and 20 and 21.1% respectively

in sheep (**Figure 3.4**)
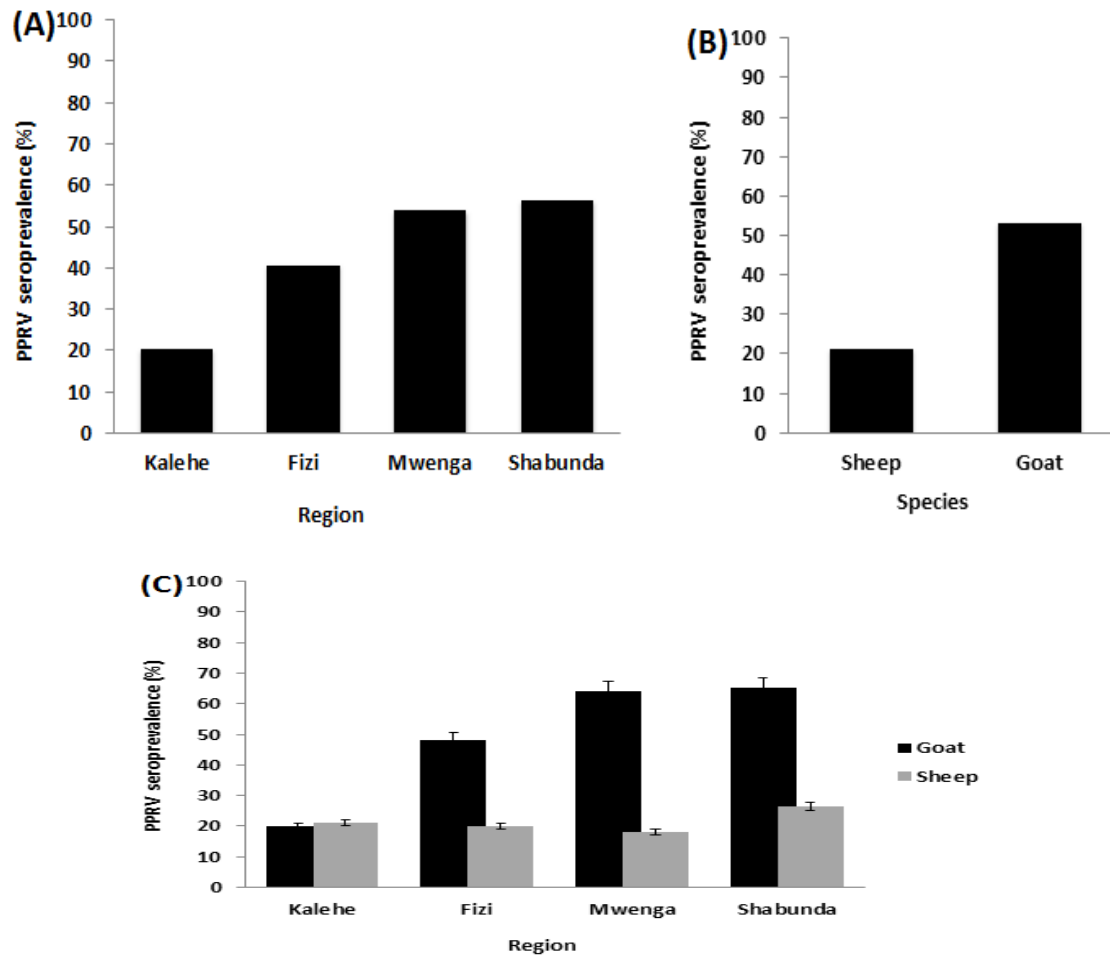
Figure 3.4: Overall PPRV-specific antibody prevalence in South Kivu. **(A), PPRV antibody prevalence in Kahele, Fizi, Mwenga, and Shabunda; (B), PPRV antibody prevalence in goats and sheep and (C) Comparative seroprevalence of *Peste des Petits Ruminants* in goats and sheep.**

3.3.2 Maps of PPRV seroprevalence in South Kivu

Shabunda, Mwenga recorded higher PPRV seroprevalence as indicated in red color in the pie based Map compared to Fizi and Mwenga (**Figure 3:5A**). The PPR seroprevalence from both goats and sheep varied between 56.5% to 20.3% from red (Shabunda), orange (Mwenga) to gold (Fizi and Kalehe). Only a few regions with very small area coverage including Uvira, Walungu, Kabare and Idjwi represented in yellow are considered now free from the disease, since no PPR outbreaks have been reported there. This arouses a need to screen animals for PPR in order to cross check and confirm the epidemiological status. However, these regions are considered to be at risk because they are sharing borders with PPR infected regions (**Figure 3:5B**). In most cases the PPRV seroprevalence is high in Mwenga colored with red compared to other regions. However, considering the PPRV seroprevalence for goats and sheep separately, the more dark the color is in the different regions the higher the PPRV antibody prevalence was observed. Kalehe region represented in orange color recorded a high sheep PPRV prevalence compared to Fizi and Mwenga regions colored in gold (**Figure 3:5 C and D**).
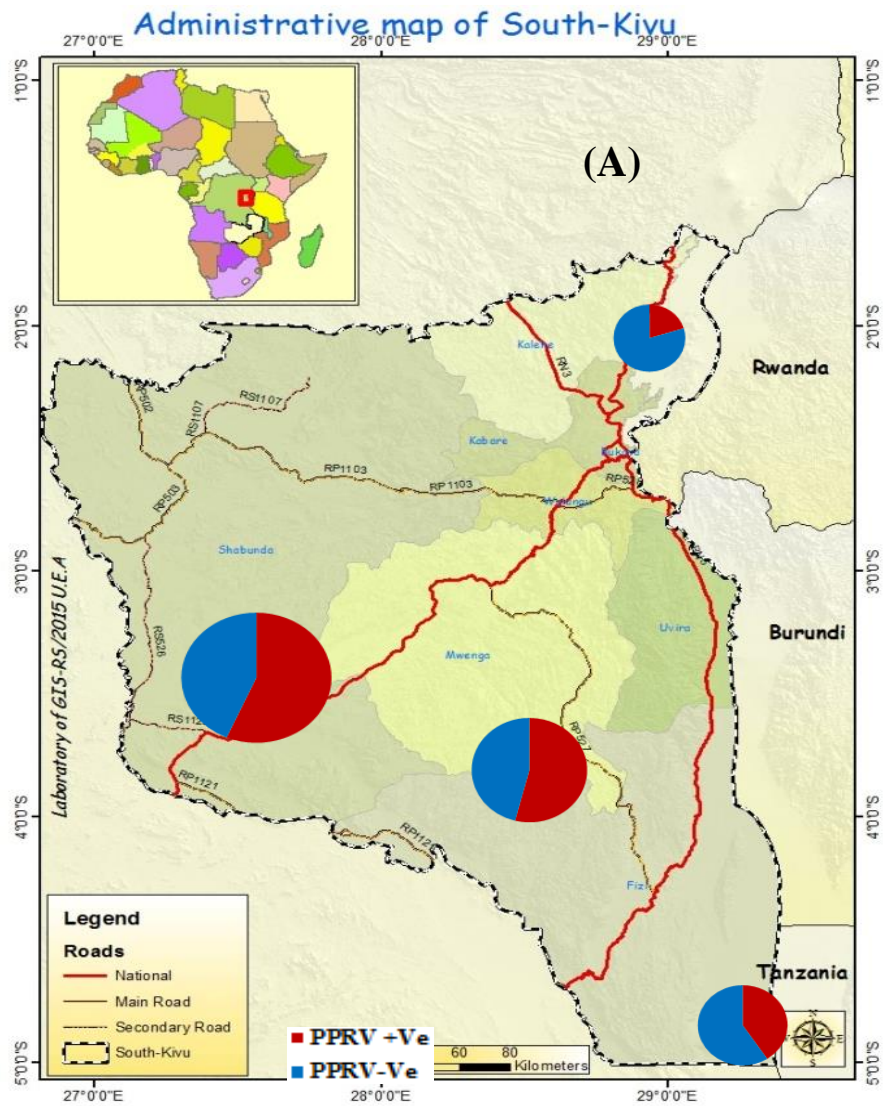
Figure 3.5: PPRV Seroprevalence maps in South Kivu. (A) PPRV seroprevalence pie chart based map in South Kivu, D R Congo; (B) Overall PPRV seroprevalence map in South Kivu; (C) PPRV seroprevalence map in Sheep in South Kivu; (D) PPRV Seroprevalence map in goats in South Kivu.

(B)

Legend

Overall PPR seroprevalence in goats and sheep(%)

- 0.0
- 0.1 - 20.3
- 20.4 - 40.8
- 40.9 - 54.0
- 54.1 - 56.5

Kalehe

Idjwi

Kabare

Shabunda

Walungu

Mwenga

Uvira

Fizi

0   15   30   60   90
Kilometer

(C)

Legend

PPR seroprevalence in sheep (%)

- 0.0
- 0.1 - 18.2
- 18.3 - 20.0
- 20.1 - 21.0
- 21.1 - 26.3

0   15   30   60   90
                              Kilometer

(D)

Legend

PPR seroprevalence in Goat (%)

- 0.0
- 0.1 - 20.0
- 20.1 - 48.2
- 48.3 - 64.1
- 64.2 - 65.2

0 15 30   60   90
Kilometer
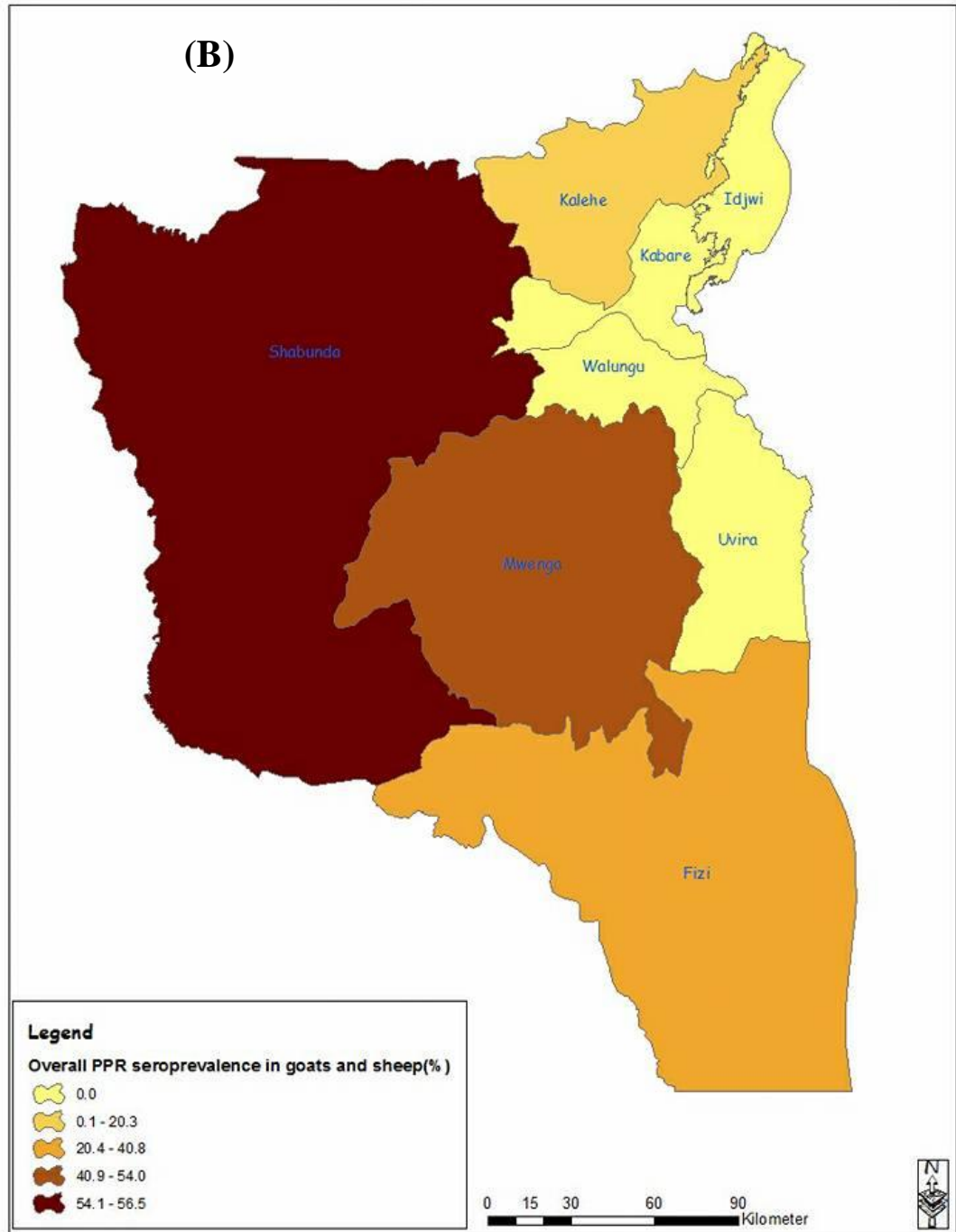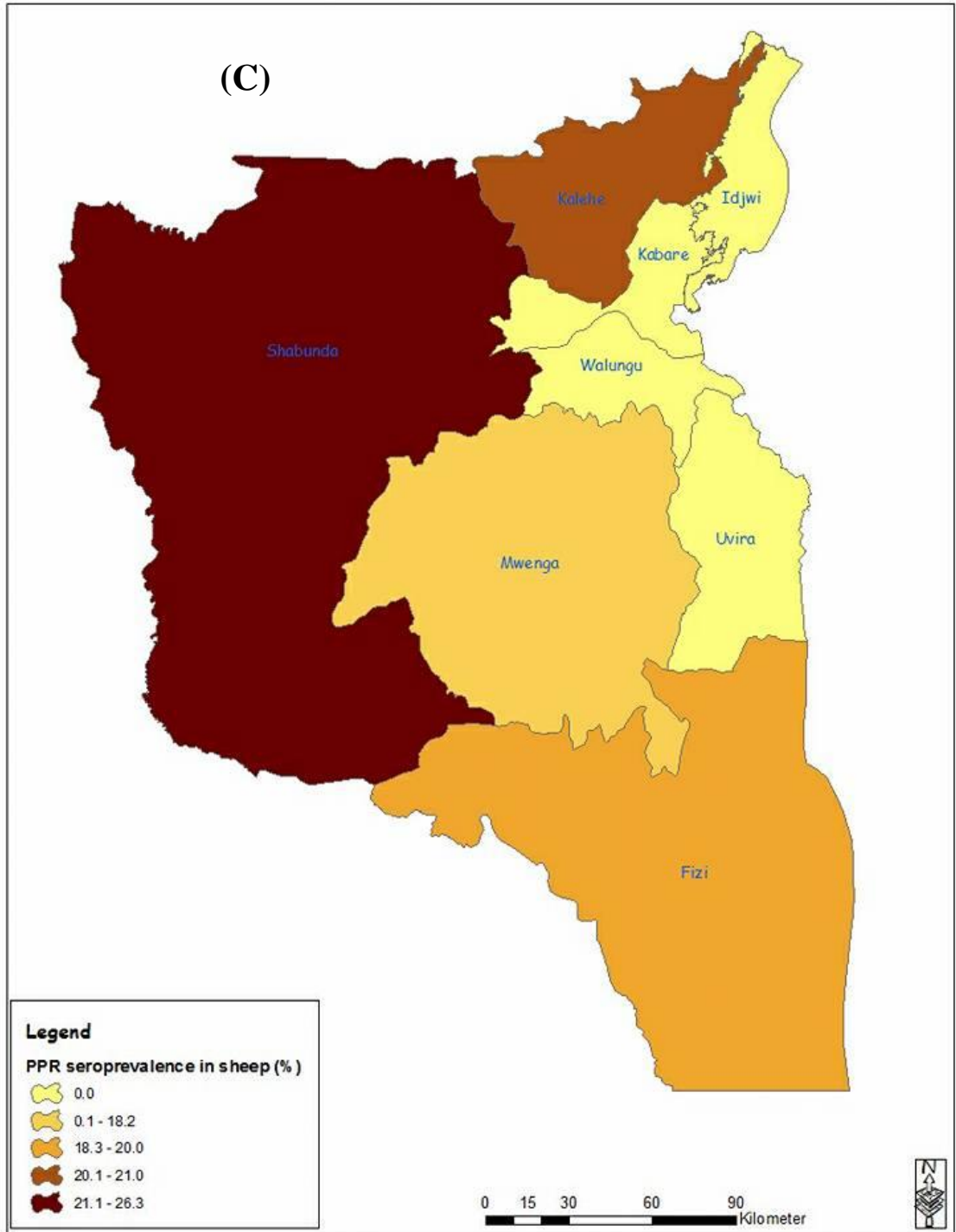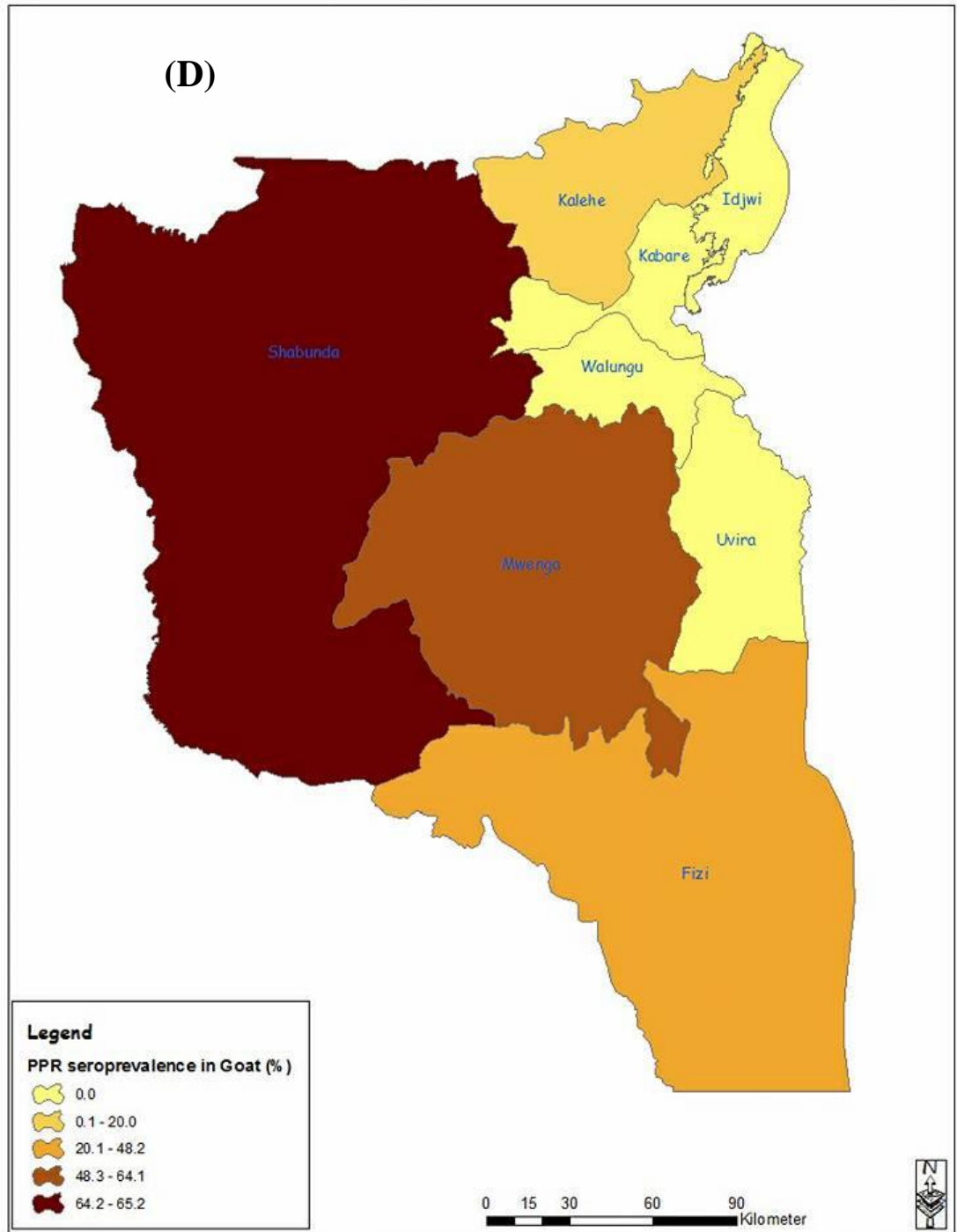
3.3.3 Potential PPRV risk factors

3.3.3.1 Univariate risk factor analyses for PPR sero-positivity

Over twenty risk factors were assessed using one structured questionnaire for every sampled herd. Eleven different factors were found to be associated with PPR seroprevalence (pv≤0.05) (**Table 3.1**). In univariate analysis when analyzed by Chi-square (χ2), Shabunda region was found to have the highest prevalence (56.5%). PPR was found to be more prevalent in goats (56.5%) compared to sheep and in exotic breeds (90.4%) compared to local ones. Female animals were more affected with PPR (53.9%) compared to males. Habitual weather condition in the farms was also associated with PPR antibody prevalence. Farms that are located in high wind speed areas had a higher PPR antibody prevalence (55.12%) compared to those located in low wind speed areas. High PPR seroprevalence rates were registered in herds where animals were sharing water points (75.3%) compared to herds where animals were not sharing water points . Regarding age groups, animals older than 12 months had the highest prevalence of PPRV antibodies (93.6%). Animals reared in communal grazing system had higher prevalence (73.5%) of PPR antibodies. Animals coming from a herd which has intermingled with animals from other herds had high PPR seroprevalence (91.4%). Animals raised in mixed flocks (goats reared together with sheep) had high (84.2%) PPR prevalence, compared to animals that were kept not mixed with other species (mono flocks). Regarding the herd size, animals originating in a herd of more than 10 animals recorded high PPR seroprevalence (96.2%) compared to animals from a small herd (less than 10 animals).

Table 3.1: Serological status of PPR antibodies based on associated determinants in goats and sheep using Chi-square (χ2) test.

Variable	Levels/ Description	No. of tested animals	No. of +ve animals	Seroprevalence (%)	χ2	df	*p-value*
Region	Shabunda	85	48	56.5	24.039	3	0.001*
	Mwenga	100	54	54			
	Fizi	76	31	40.8			
	Kalehe	59	12	20.3			
Species							
	Goat	240	128	53.4	26.394	1	0.000**
	Sheep	80	17	21.3			
Breed							
	Exotic	115	104	90.4	163.085	2	0.001*
	Local	125	25	20			
	Cross breed	80	16	20			
Sex							
	Female	228	123	53.9	24.93	1	0.002*
	Male	92	22	23.9			
Wind- speed							
	High	185	102	55.12	17.336	1	0.000**
	Low	135	43	31.85			
Water source							
	Water Shared	178	134	75.3	167.929	1	0.000**
	Not sharing	142	10	7.1			
Age(months)							
	>12	109	102	93.6	187.264	2	0.000**
	5-12	114	33	28.95			

	<5	97	10	10.3			
Grazing systems							
	Communal	181	133	73.5	149.687	1	0.001*
	Zero-grazing	139	12	8.62			
Farm-to-farm animal exchange							
	Animal exchange	139	127	91.4	241.835	1	0.001*
	No animal exchange	181	18	9.9			
Herd composition							
	Goat flock	153	47	30.7	135.569	2	0.001*
	Mixed flock	114	96	84.2			
	Sheep flock	53	2	3.8			
Herd size							
	>10 animals	53	51	96.2	198.8	2	0.002*
	5-10animals	93	73	78.5			
	<5 animals	174	21	12.1			

Chi-square ($\chi 2$) test, df (degree of freedom), p-value (significance level), +ve (positive samples), < less sign, > Greater sign, *significant, ** highly significant

3.3.3.1.1 Flock type and size

The PPR seroprevalence in animals from flocks having more than 10 animals each was high, 96.2% (51/53), followed by the animals from flocks having 5-10 small ruminants with 78.5% (73/93). A low PPR antibody prevalence of 12.1% (21/174) was found in animals from flocks with less than 5 animals (**Figure 3.6A**). The PPRV seroprevalence was 30.7 % (47/153) in goat's mono flocks, 84.2% (96/114) in mixed flocks (goats-sheep) and 3.8% (2/53) in sheep mono flock, 96.2% (51/53) as shown in **Figure 3.6B**
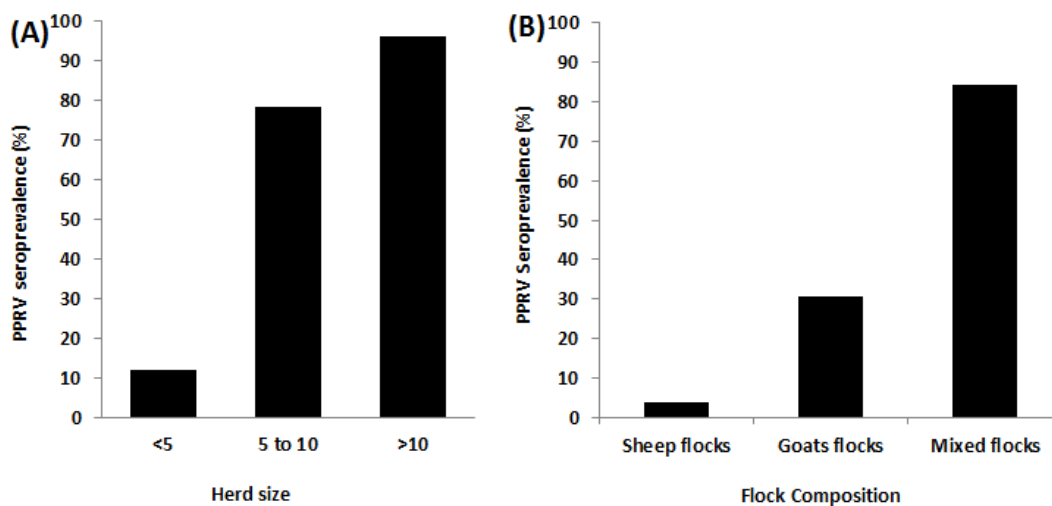
Figure 3.6: PPRV seroprevalence per herd size and type. (A) PPRV seroprevalence according to herd size, (B) PPRV seroprevalence according to herd composition.

3.3.3.1.2 Breed

Exotic animals were more PPRV seropositive, with 90% (104/115). The PPRV antibody prevalence in local breed was 20% (25/125) and 20% (16/80) in cross-breed (**Figure 3.7**).
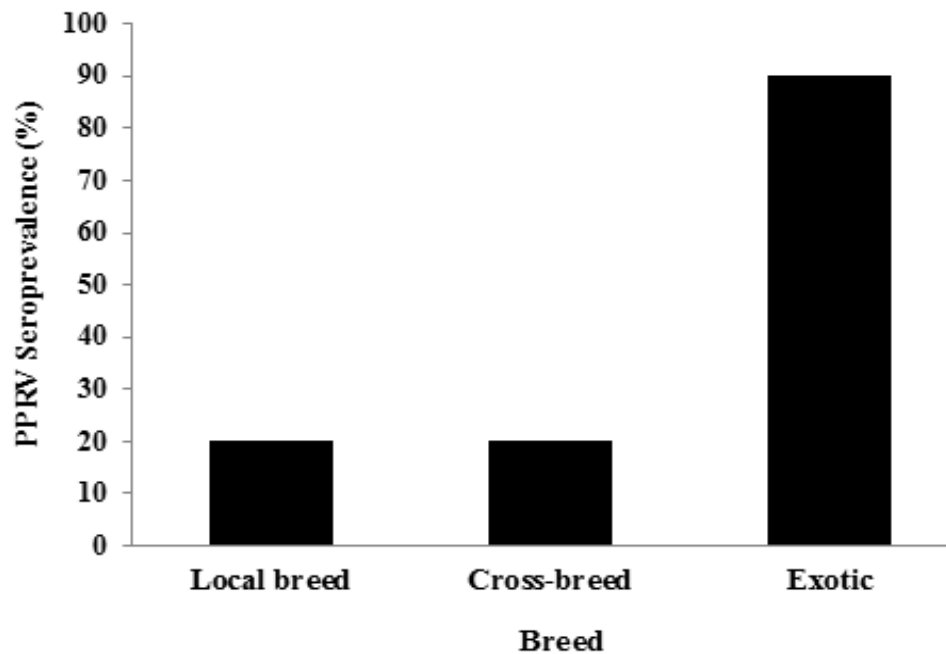
Figure 3.7: Breed based PPRV seroprevalence (local, cross and exotic breed).

3.3.3.1.3 PPR antibody seroprevalence by Sex

The PPR antibody overall prevalence differed between male and female animals. The current results found that female goats and/or sheep had higher 53.9 % (123/228) PPR seroprevalence compared to male goats and/or sheep which was 23.9% (22/92) (**Figure 3.8**).
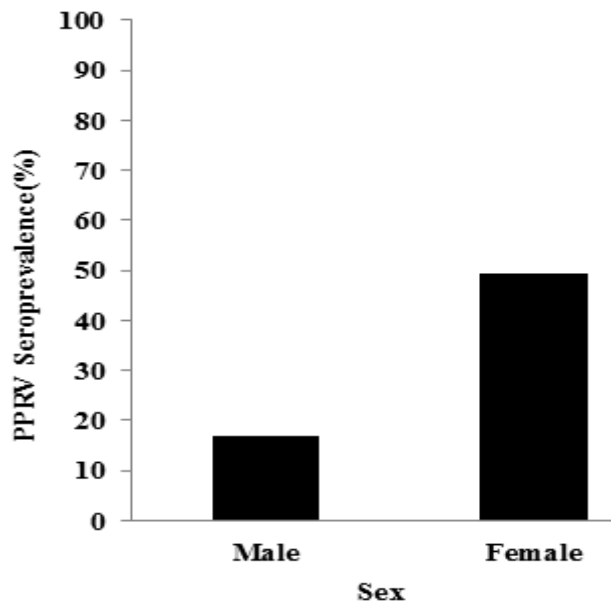
Figure 3.8: Sex based PPR seroprevalence in goats and sheep in South Kivu

3.3.3.1.4 PPR seroprevalence by farming and water system, age and animal exchange

On one hand, animals raised in communal grazing systems had higher PPRV seroprevalence at 73.5% (133/181) compared to those kept in zero-grazing system at 8.6% (12/139) (**Figure 3.9A**). On the other hand, PPRV antibody prevalence was higher in animals which were sharing the water point 75.3% (134/178) compared to those which were not sharing water point , which was 7.1% (10/142) (**Figure 3.9B**). The PPRV seroprevalence varied among the three age-groups. It was higher in animals aged more than 12 months 93.6% (102/109) and lower in young animals less than 5 months old with at 10.3% (10/97) (**Figure 3.9C**). Exchange of animals from farm-to-farm led

to significant increases of PPRV antibody prevalence in the study area, with an overall

PPRV seroprevalence of 91.4% (127/139) (**Figure 3.9D**).
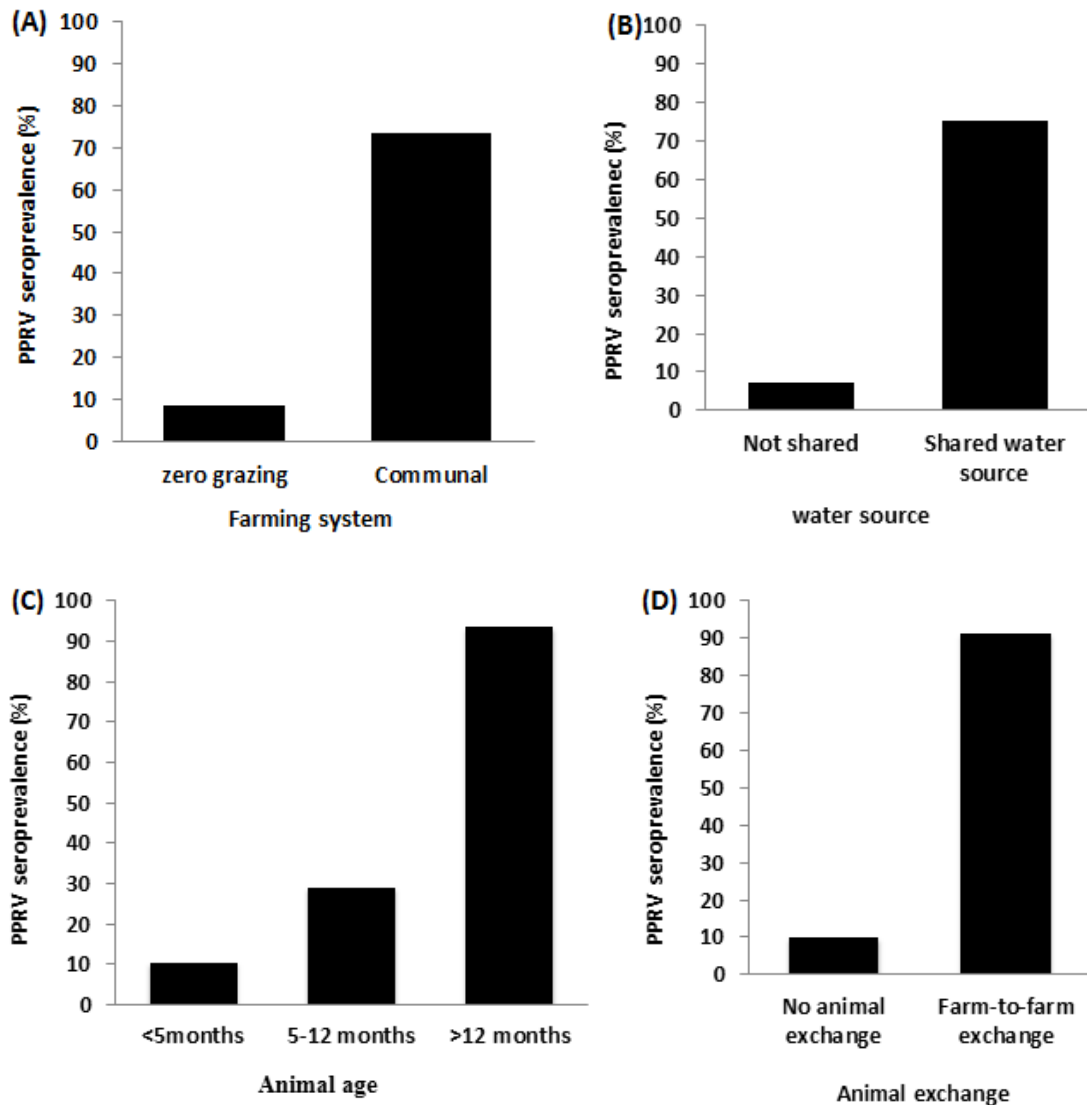
Figure 3.9: Farming system and management based PPRV seroprevalence. (A) Grazing system based PPR seroprevalence, (B) watering system source based PPR antibody prevalence, (C) PPR seroprevalence per age, (D) Farm-to-farm animal exchange based PPRV seroprevalence.

3.3.3.2 Multivariate risk factor analyses for PPR sero-positivity

The association between the animals with PPR exposure and the 11 potential risk factors found through univariate analysis was assessed in a multivariate analysis which revealed using logistic regression; with confidence interval of 97.5% and differents levels of *p-values* ranging from 0.001 to 0.05. Sheep and goats from three territories, including Shabunda, Mwenga and Fizi, were more exposed to PPR with respectively (OR 0.22; OR 0.57 and OR 0.08). Goats were found to be at higher risk than the sheep for seropositivity against PPR (OR 0.84). Exotic breeds were more at risk of PPR seropositivity compared to local (OR 0.98) and cross breeds (OR 36.5). Multivariate analysis identified sex (female) as a significant risk factor for PPR seroprevalence (OR 0.34) as shown in **table 3.2.** Moreover, goats and sheep aged with age above 12 months were at a higher risk of seropositivity of PPRV compared to young (OR 3.94) with age between 5-12 and goats and sheep (OR 58.4) with age lower than 5 months old. Sheep and goats reared in communal/open grazing systems and those sharing water points were more likely to be PPRV seropositive compared to animals kept in zero-grazing (OR 0.10) and those not sharing water points (OR 5.27). Farms that exchanged animals frequently were more exposed to PPRV infection (OR 4.60) and farms with large herd size (≥5 goats and sheep) were likely to be PPRV seropositive (OR 0.04) in farms with 5-10 goats and sheep and (OR 2.71) in goats and sheep coming from a herd size greater that 10. Goat flocks were found at higher risk than mixed flocks (goats and sheep) for seropositivity against PPR (OR 3.95) and sheep flocks (OR 0.06) (**Table 3.2**).

Table 3.2: Multivariate analysis for the association between PPR status and the potential risk factors

Variable	Levels	Odds Ratio	[97.5% CI]	p-value
Species	Goat	-	-	
	Sheep	0.84	0.214 3.384	0.0073387**
Region	Fizi	-	-	
	Kalehe	0.08	0.013 0.394	
	Mwenga	0.57	0.135 2.300	0.0642192 .
	Shabunda	0.22	0.039 1.037	
Breed	Cross breed	-	-	
	Exotic	36.51	16.56 87.67	0.005**
	Local breed	0.98	0.488 2.027	
Sex	Female	-	-	
	Male	0.34	0.093 1.178	0.0950247 .
Sharing water source	Not sharing	-	-	
	Sharing	5.27	1.879 18.739	0.003628 **
Age (months)	<5	-	-	
	5-12	3.94	1.091 16.04	
	>12	58.42	13.82 316.34	2.630e-07 ***
Grazing system	Communal	-	-	
	Zero-grazing	0.10	0.026 0.294	0.0001004 ***
Farm-to-farm animal exchange	No	-	-	<2e-16 ***
	Yes	4.60	0.40 11.47	
Herd size	<5	-	-	
	5-10	0.04	0.02 0.07	< 2.2e-16 ***
	>10	2.71	1.96 3.81	
Flock type	Sheep flock	-	-	
	Goats flock	0.06	0.004 0.605	0.0177310 *
	Mixed flock	3.95	1.297 12.913	

Signif. Codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1, CI: 97.5% confidence interval

3.3.4 Clinical signs and gross pathological findings

The main clinical signs observed in PPR-suspected animals were diarrhea, serous mucopurulent oculo-nasal discharges, and lesions around the muzzle, dullness, fever and cutaneous nodules (**Figure 3.10**).

Post mortem findings (in black arrow) included haemorrhages in large intestine, internal cutaneous nodules, congestion and haemorrhages in lungs and on liver (**Figure 3.11**).

Figure 3.10: Clinical signs of PPR-suspected animals in South Kivu in arrow. Animal with diarrhoea (A), ocular discharge (B), nasal discharge (C) cutaneous nodules (D).
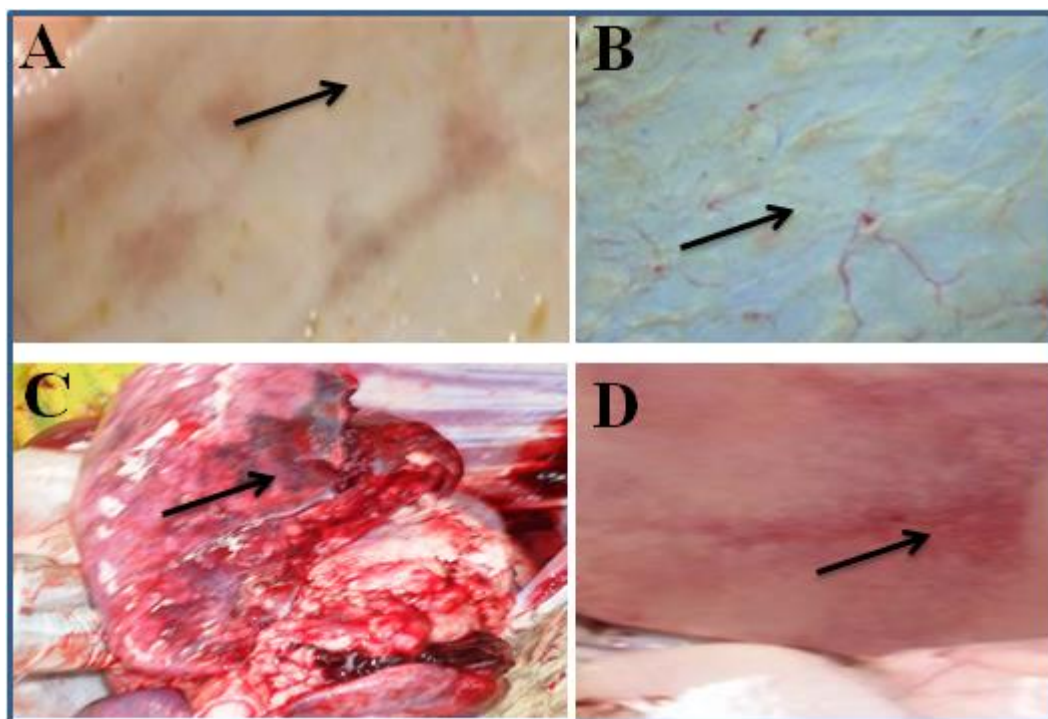
Figure 3.11: Necropsy of PPR-suspected animals. Large intestine haemorrhages. (A), internal skin nodules (B), Hemorrhagic and congested lung (C), Haemorrhages liver (D).

3.4 DISCUSSION

In Africa, *Peste-des-petits-ruminants* was first recognized as a contagious "rinderpest-like" condition in goats. Previous studies from Kerur *et al*. (2008), Zhao *et al.* (2010), Banyard *et al.* (2010) and SADC (2012b) confirmed that PPR was first recognized in Western Africa. It later spread to Asia and then was introduced to Ethiopia and Sudan (Kwiatek *et al.,* 2007; Muse *et al.,* 2012). References show that transboundary uncontrolled animal movement might be the source of introduced of the disease in African countries (Tylor, 1979; Muse *et al.,* 2012).

The South-Kivu region of the D. R. Congo was infected since 2008, when PPR outbreaks based on clinical signs, were reported for the first time, but no laboratory diagnosis had been done to confirm the cases. The current study findings showed that PPR antibody seroprevalence was heterogeneous across sampling regions and that recorded PPR antibody seroprevalence was at 45.3% overall. Previous studies from Turkey showed a seroprevalence of 45.4% (Ozkul *et al., 2002*), while that of 31.0% was registered in Tajikistan in Central Asia (Kwiatek *et al*., 2007). In different studies conducted in Sudan, high levels of PPRV seroprevalence including 50.6%, 62.8% and 61.8% were observed (Abubakar *et al., 2009*; Veterinary Record, 2012; FAO, 2015).

In this study, it was found that goats had higher PPR-Antibody prevalence compared to sheep (**Table 3.1** and **3.2**; pv=0.045). These results are supported by several studies from Ismail and House (1990), Zhao *et al*. (2010) and Kayunze *et al.* (2012) showing that goats are more susceptible to the disease than sheep. This might be due to the natural immunity of sheep compared to goats and a higher susceptibility to the virus for the latter. It may also be due to the nature and virulence of the strains circulating in different regions, or due to differences in management systems of small ruminant flocks (Rossiter, 2004; Abubakar *et al*., 2009; Veterinary Record, 2012; FAO, 2015). Further, because PPR virus and the now-eradicated rinderpest virus are cross-protective, the rapid expansion of the PPR virus within endemic zones and into new regions may be due to the disappearance of the cross-protection previously conferred by natural rinderpest infections of small ruminants and/or the practice of using rinderpest vaccine to prevent small ruminant infection with PPR virus in certain endemic areas (Haroun *et*

al., 2002; Dhar *et al.,* 2002; Luka *et al*., 2012; FAO and OIE, 2016). Thus, PPR virus has managed to cause severe epidemics, or even pandemics, in larger small ruminant populations in an increasingly expanding area of the developing world. Shabunda and Mwenga regions had the highest PPR antibody prevalence (**Table 3.1**; **Figures 3.4 and 3.5**).This could be explained by the fact that Shabunda and Mwenga are neighboring counties. It could also be due to the uncontrolled trans-border movement of ruminants between these territories and the Kalima and Maniema regions where PPR outbreaks have been reported several times in the past (Anderson *et al*., 1991; SADC, 2011).

This study also showed that the PPRV seroprevalence varied among the three age-groups; the older animals were found to be more PPRV seropositive compared to the younger ones (**Table 3.1; Figure 3.9C**). Seropositivity in the young ones could have been due to the maternal antibodies as tend to have maternal antibodies up to six months if the parents were immune to PPR (Rossiter, 2004; Abubakar *et al*., 2009 Veterinary Record, 2012; FAO, 2015). There is also a possibility that some animals in the age-group under one year may have been exposed to PPRV but survived and seroconverted (Muse *et al.,* 2012).

The PPRV seroprevalence reported in this study was lower in animals kept in zero-grazing system and higher in those kept in communal grazing systems. Moreover it was higher in cases where animals were sharing water sources. It is likely that the discharge of PPRV from infected animals contaminated water and feed, thus spreading the disease to non-infected animals. These results are similar to those of other reports, showing that

PPR infection between animals occur during communal grazing and housing (Haroun *et al*., 2002; Osman *et al.,* 2009; Muse *et al.,* 2012; Abdalla *et al.,* 2012).

In this study, exchange of animals from farm-to-farm appeared to contribute to PPR distribution. This led to a highly significant increase of PPRV antibody prevalence in South Kivu (**Table 3.1** and **Figure 3.9D**). This finding is in agreement with Haroun *et al*. (2002), Ularamu *et al*. (2012) and Kgotlele *et al*. (2014) who ranked the introduction of non-tested animals as a risk factor that increases the probability of disease transmission from infected to non-infected animals.

In this study, data from flock composition and size indicated that flocks of goats alone and those of mixed goats and sheep developed higher PPRV antibody seroprevalence compared to flocks with sheep alone. Moreover, herds with more than 10 animals demonstrated higher PPRV seroprevalence when compared to flocks with herd size of five to 10 animals (**Table 3.1; Figure 3.6**). These observations are in agreement with previous findings where sheep were found to be less severely affected by PPRV than goats, probably due to the better natural immune resistance to PPRV in sheep (Haroun *et al*., 2002; Osman *et al*., 2009; Saeed *et al.,* 2010; Abdalla *et al.,* 2012). Large flock size (more than 10 animals per flock) and mixed flocks (goats-sheep), were more exposed to PPR infection making the control and surveillance of individual animals difficult. The current study also revealed that females are more significantly affected by PPRV than males (**Table 3.1**). This is in agreement with findings in Sudan (Rossiter, 2004; Abubakar *et al*., 2009; Veterinary Record, 2012).   In Democratic Republic of

Congo, this observation can be explained by the fact that, with respect to current breeding system in the region, and especially in South Kivu, female animals are kept for a longer period of time for reproduction purposes. Hence, Abubakar *et al*. (2011) showed that the sex ratio between female and male goats or sheep was extremely high in favor of females with a ratio of 19 to 1, respectively.

The laboratory confirmation is necessary for accurate definitive diagnosis although postmortem and clinical examinations may be used for presumptive PPR diagnosis. In the outbreak understudy, the clinical signs, postmortem findings and epidemiological observations clearly indicated the presence of PPR virus. However, oculo-nasal discharges and haemorrhages and congestions of lung were very mild as described by Wamwayi *et al*. (1995); Abubakar *et al*. (2011) and Kgotlele *et al*. (2014). Although, PPR is a viral disease, it is complicated by secondary bacterial infection(s). Several findings from Abdollahpour *et al*. (2006) and Soumare (2013) concluded that lung lesions in PPR, i.e: interstitial pneumonia, are generally complicated by secondary infections.

This study has shown that there is wide variation in the prevalence of PPRV and its associated determinants among goats and sheep in different regions of South Kivu in the Eastern part of the Democratic Republic of the Congo.

CHAPTER FOUR: MOLECULAR CHARACTERIZATION OF *PESTE DES PETITS RUMINANTS* VIRUS ISOLATES IN SOUTH KIVU PROVINCE

4.1 INTRODUCTION

Peste des petits ruminants is a serious contagious viral epizootic disease of sheep, goats, springbuck, gazelles and impala and camels. The disease is transmitted by peste-des-petits-ruminants virus (PPRV), which is a negative sense RNA virus from the *Paramyxoviridae* family, sub-family *Paramyxovirinae* and *Morbillivirus* genus. Important pathogens of this family include canine distemper virus (CDV), rinderpest virus (RPV) and measles virus (MV) (Luka *et al.,* 2012). Morbidities due to PPR go up to 100% and mortality rates between 50 and 90% (Rossiter, 2004; Banyard *et al.,* 2010). Goats are more susceptible to the disease compare to other species and may die in ten days of exposure to PPRV in several outbreaks (Zhao *et al.,* 2010; Abubakar *et al*., 2011; Muse *et al.,* 2012a). In Africa, PPR was first recognized as a contagious "rinderpest-like" condition in goats which originated in Western Africa, spread to Asia and then to Sudan and Ethiopia (Swai *et al.,* 2009; Kaukarbayevich, 2009). The report from SADEC (2012a and b) showed the presence of PPR in the South-Kivu province of D.R. Congo in 2008 for the first time, when outbreaks based on clinical signs was reported. However, no molecular laboratory based diagnosis was done to confirm the cases and understand genetic nature of the virus circulating in this region or country. Recent studies on evolutionary and epidemiological dynamics of PPRV have been possible through the analyses of partial or full nucleoprotein (NP) and fusion (F) gene sequences of the virus. All the four previously described PPRV lineages (I-IV) diverged

from their common ancestor during the late 19th to early 20th century. During recent years, in several African countries lineage IV-PPRV have been detected in several African countries and seem to spread efficiently in the African continent according to Kwiatek *et al.,* (2011), Cosseddu *et al.* (2013), Maganga *et al.* (2013) and Muniraju *et al.* (2014). Among the four known lineages, PPRV-IV showed pronounced genetic variation across the region (Padhi and Ma, 2014). Previous reports showed an extent of geographic specificity of the four PPRV lineages. Several authors classified restrictedly lineages I to western Africa, lineage II to Central Africa, while lineage III was classified to the Arabian Peninsula and eastern Africa (Banyard *et al.*, 2010; Albina *et al.*, 2013; Libeau *et al.,* 2014). The lineage IV, which is also referred to as the Asian lineage, had a wide geographic coverage ranging from Southeast Asia to the Middle East, and had more recently expanded into northern Africa (Libeau *et al.,* 2014). Previous studies done by Kwiatek *et al.* (2011), Abubakar *et al.* (2012) and Maganga *et al.* (2013) showed that lineage IV was not only the predominant lineage across the region but also gradually replaced lineage III in some parts of Africa.

Although the reason why lineage IV is more widespread than other lineages is unknown; one may only speculate that lineage IV has a selective advantage over other lineages. Under such circumstances, some amino acid residues in the PPRV-IV proteins are expected to evolve adaptively (Singh *et al.*, 2004; Padhi and Ma, 2014). This study was aimed at determining the PPR prevalence, characterizing genetically the PPR virus strains by establishing their phylogenetic relationship compared to other PPRV isolates

in Africa and other parts of the world based on nucleoprotein, fusion and haemagglutinin gene sequencing.

4.2 MATERIALS AND METHODS

4.2.1 Study area

This was as given in Section 3.2.1 above

4.2.2 Sample size

A total of 150 samples including whole blood (80), swabs (53) and tissues (17) were collected from different animals (goats and sheep) that were presenting PPR-suspected clinical signs as described in Chapter 3, section 3.3.4 and **figures 3.9** and **3.10.**

4.2.3 Sample collection and processing

The samples were collected from either live PPR suspected animals (goat and sheep) or dead after post-mortem diagnosis.

4.2.3.1 Whole blood

Approximately 4 ml of whole blood sample was taken from the PPR-suspected animals in the jugular vein using a needle and blood collected into vacutainer tubes containing anticoagulant, EDTA (BD Biosciences, Franklin Lakes, USA). Each tube was labelled with codes describing the characteristics of specific animal and flock/farm. An aliquot of each sampled whole blood was kept as a backup on Whatman ®FTA cards that simplified the handling and processing of nucleic acids in case the whole blood transported in tube in the cold chain system failed to work properly.The buffy coat was

obtained by centrifuging 500 µl histopaque®-1077 (Sigma-Alderich, St. Louis, USA) layered with 1 ml whole blood at 400 x g at 4ºC for 30 minutes. The opaque interface (buffy coat) was carefully moved into an antiseptic microfuge tube and kept at -80ºC until the extraction of RNAwas done, using both phenol/chloroform protocol of Sambrook *et al*. (1989) and RNeasy®Plus mini-kit, following manufactures' instructions. The upper layer was discarded.

4.2.3.2 Nasal and ocular swabs

Nasal and ocular swabs were collected from the animals and stored in 3 ml BD Universal viral transport medium (BD Biosciences, Franklin Lakes, USA). They were then vortexed to dislodge any cells from the swabs and centrifuged for five minutes at 8000 x g at room temperature. The supernatant was decanted into disinfected microfuge tube and kept at -80ºC until the extraction of RNA was done.

4.2.3.3 Tissue collection and preparation

Tissues collected from sacrificed or dead animals were: intestines, lymph nodes, liver and lungs; those from the same animal were pooled together. Tissues were stored in RNA-later® (ThermoScientific, USA) after collection until they were shifted to the International Livestock Research Institute (ILRI) laboratories in Kenya where they were stored at -80°C until when RNA extractions were done. Approximately 250 mg of tissue sample was ground and homogenized in 2.5 ml of Gibco® F-12 Mixture Nutrient to make a tissue suspension of 10% which was then centrifuged at the room temperature for five minutes at 8000 x g and put in storage at -80ºC until the extraction of RNA.

4.2.4 Sample analysis

4.2.4.1 RNA extraction

Aliquot supernatants from ground and homogenized tissues, nasal and ocular swabs were used for RNA extraction using the kit of RNeasy®Plus mini as per the manufacturer's protocol (**Appendix 3**). Using the buffy cost, the viral RNA was recovered, homogenized. The RNA from both plasma and the whole blood was extracted using the kit of QIAamp Viral RNA Mini extraction according to the manufacturer's protocol (**Appendix 4**). Briefly, samples were lysed using a lysis buffer followed with protein precipitation using ethanol. The lysate was then passed through a Qiagen column followed by washing and cleaning of bound RNA. Afterwards, the RNA was eluted with free water RNase. Finally, the RNA extracted was kept at -80ºC until amplification was done.

4.2.4.2 RNA quantification and quality testing

Extracted RNA samples were checked for concentration, integrity and purity using nanodrop 2000, Bio analyzer (High Sensitivity RNA ScreenTape® 2200 TapeStation following the protocol detailed in **appendix 5**), Qubit® RNA High Sensitivity (HS) and Broad range (RB) Assays using the Qubit® Fluorometer protocol described in **appendix 6**). The Qubit assay kit used was from ThermoFisher Scientific (Cat. number: **Q32855**). Using the buffer provided by the kit, the assay reagent from was diluted and RNA samples. Then the concentration results were read using the Qubit® Fluorometer. To reduce the risk of RNA being degraded by RNases, an agarose gel electrophoresis of 1.2% with TAE 0.5x buffer treated with 0.1% diethyl pyrocarbonate (DEPC) was used.

The buffer was used to evaluate the degradation and integrity of the extracted RNA. The same mixed buffer was used to clean the electrophoresis tank, comb and other materials used.

4.2.4.3 Detection of PPRV by RT-PCR

The synthesis of DNA from an RNA template, via reverse transcription was carried out using an in virtogene reagent, SuperScript™ II Reverse Transcriptase (Cat. No. 18064-022) following the manufacturer's procedure (**Appendix 7**). Reverse transcription polymerase chain reaction was runned out in GeneAmp PCR System 9700. On one hand, superScript™ II reverse transcriptase (Cat. No. 18064-022) was used following the manufacturer's protocol (**Appendix 8**) for RT-PCR amplification of the First-Strand cDNA Synthesis. On the other hand the master mix as shown in **Table 4.1** was prepared using the DreamTaq Green PCR Master Mix (2X), a one-step RT-PCR kit (Catalog number: K1081,ThermoFisher Scientific) using both PPRV specific primers that were designed using CLC workbench software and BLAST online tool and the published ones (**Table 4.2**). All reactions were run with the stain of Nigeria_75/1 vaccine as a positive control with nuclease-free water as the negative control. Briefly, in a 20µl reaction volume containing approximatively 50 ng/µl of total RNA, a reverse transcription was carried out. Each tube contained: 1 µl of 50–250 ng random hexamers, 1 ng to 5 µg total RNA, 1 µl dNTP-Mix (10 mM) ,12 µl of sterile. The mixture was heated to for five minutes at 65° C and quickly kept on ice-cold before a brief centrifugation of 30 seconds at full speed (14,000rpm). Approximatively, 4µl of 5X-First-Strand buffer, 2 µl (0.1 M DTT) and 1 µl of RNaseOUT™ (40 units/µl)

were added; the tube mixture were gently mixed before incubated for 2 minutes at 42°C. In addition, 1μl (200 units) of SuperScriptTM II RT was then added and mixed with pipettes before a long period of incubation for 50 minutes at 42° C. To inactivate the reaction, heating for 15 minutes at 70°C was done. The designed and ordered primers were generated in a conserve region after multiple alignments of six PPRV strains (*Ethiopia 1994, Ethiopia 2010, Ghana 2010, Morocco 2008, Uganda 2012, Nigeria 75*) that covered all the four known lineages. Own primers were designed with long fragments (NL, P, FL and HL) because some of the published primers used, including F, NP3-NP4, F, H, UP, qPCR (**Table 4.2**), were shorter and some of the regions could not be covered by PPRV isolated in this study.

Table 4.1: Reverse transcription polymerase chain reaction mix components.

No.	Component	Volume (μl)
1.	cDNA template (Diluted 1:50)	1
2.	10μM Forward primer (10pmoles/ μl)	0.5
3.	10μM Reverse primer (10pmoles/ μl)	0.5
4.	Nuclease-free water	3
5.	Master Mixt Dream Taq green	5
	Total volume per reaction	**10**

Table 4.2: *Peste des petits ruminants* **virus specific primers**

No	Name	Gene	Location (bp)	Sequencing	Amplicon size	TM (°C)	Reference
1.	NP3-forward	Nucleoprotein (NP)	1232-1255	5'- TCTCGGAAATCGCCTCACAG -3'	351bp	63.3	Ularamu *et al.*, 2012; Couacy-Hymann *et al.*, 2002
	NP4-reverse	Nucleoprotein (NP)	1583-1560	5' - CCTCCTCCTGGTCCTCCAGA -3'	351bp	61.7	Ularamu *et al.*, 2012; Couacy-Hymann *et al.*, 2002
2.	NL-forward	Nucleoprotein (NP)	-	5'-ATCCGGGGGATTAAGAATGTTAT-3'	1430bp	57.3	Designed for this study by Ahadi
	NL-reverse	Nucleoprotein	-	5' -ATCTTGGCCATGGCCTGCAG -3'	1430bp	61.6	Designed_Ahadi
3.	P-forward	Phosphoprotein	-	5' - ATGGCAGAAGAACAAGCATACCATGTC -3'	1530bp	62.2	Designed_Ahadi
	P-reverse	Phosphoprotein	-	5' - TTACGGCTGCTTGGCAAGAATGGCTGT - 3'	1530bp	69.4	Designed_Ahadi
4.	F-forward	Fusion protein	777-801	5' -GAGACTGAGTTTGTGACCTACAAGC -3'	372bp	55.1	Esmaelizad *et al.*, 2011; Forsyth and Barrett, 1995
	F-reverse	Fusion protein	1124-1148	5' -ATCACAGTGTTAAAGCCTGTAGAGG -3'	372bp	54.8	Esmaelizad *et al.*, 2011; Forsyth and Barrett, 1995
5	FL-forward	Fusion protein	-	5' -	1376bp	64.3	Designed_Aha

No.	Primer	Target		Sequence	bp	TM	Source
				ATGCCAAATATAACAGCCATCGACAAT TG -3'			di
	FL-reverse	Fusion protein	-	5' -ATGACGCCAAGGGAAACACTCTAT - 3'	1376bp	62.8	Designed_Aha di
6	H-forward	Haemagglutinin	-	5'- CCGGCATGGATCTTTACAACAAC -3'	410bp	59.1	Kumar N.*et al.*, 2013
	H-reverse	Haemagglutinin	-	5' - ATGACGCCAAGGGAAACACTCTAT - 3'	410bp	58.9	Kumar N.*et al.*, 2013
7.	HL-forward	Haemagglutinin	-	5' - TGTCAACTTCATCTCCGATAAG -3'	1489bp	50.9	Designed_Aha di
	HL-reverse	Haemagglutinin	-	5' -TCAGACTGGATTACATGTTACCTC -3'	1489bp	51.6	Designed_Aha di
8.	UP-forward	Universal	-	5' -ATGTTTATGATCACAGCGGT -3'	-	49.1	Commercial Bionneer
	UP-reverse	Universal	-	5' –ATTGGGTTGCACCACTTGTC -3'	-	54.3	Commercial Bionneer
9.	qPCR-N-rev1	Virus screening	-	5' - GTTTAGCTTCCTCTGCTGTGAT -3'	<100bp	51.9	Commercial Bionneer
	qPCR-N-rve2	Virus screening	-	5' - GTTTGGCTTCCTCTGCTGTGAT -3'	<100bp	57	Commercial Bionneer
	qPCR-N-fwd1	Virus screening	-	5' -TCTCGGACAGGAGATGGTCAGA -3'	<100bp	58.2	Commercial Bionneer
10.	OligodT	cDNA maker	-	5' - NNNNNN-3'		18	Commercial Bionneer
11.	Random Hexamer	cDNA maker	-	5' TTTTTTTTTTTTTTTTTVN -3'		43.3	Commercial Bionneer

TM: Mulching temperature; bp: base pare, NL: nucleoprotein long fragment primers, FL: long fragment fusion protein primers,

HL: Long fragment haemagglutinin protein primers, P: Phosphoprotein primers, UP: universal primers

4.2.4.4 Cycling conditions

The cycling conditions for the cDNA (conversion of RNA extracted to complementary DNA) was as described in superScript™ II reverse transcriptase protocol as given in **Appendix 7** and **figure 4.1;** , the amplification conditions for all the genes having been found after an optimization of cycling and temperature conditions through a gradient PCR. Therefore, the optimal cycling conditions for all the genes amplified were similar (**Figure 4.1**).
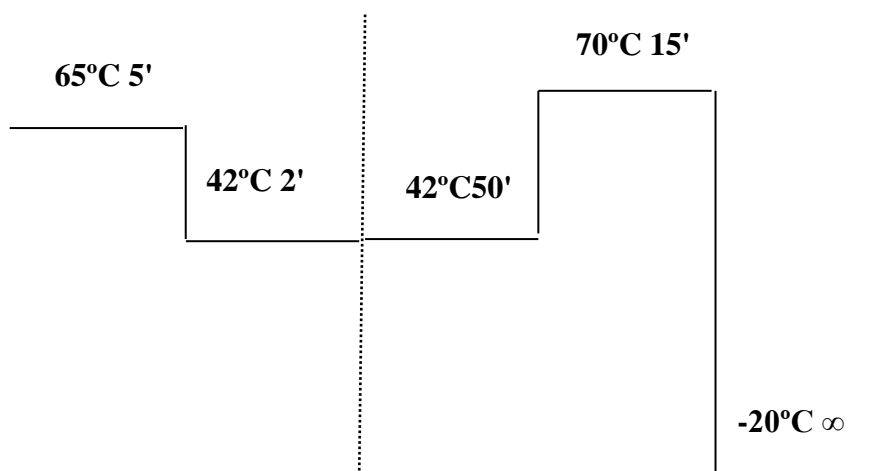
Figure 4.1: cDNA synthesis cycling condition

The cDNA amplification was done for 2 minutes at 95°C before initial denaturation followed by 35 cycles (Denaturation for 30 seconds at 94° C, annealing for 45 seconds at 55°C and elongation for 2 minutes at 72° C). A final extension for 7 minutes at 72° C followed before the amplification was stopped (**Figure 4.2**).
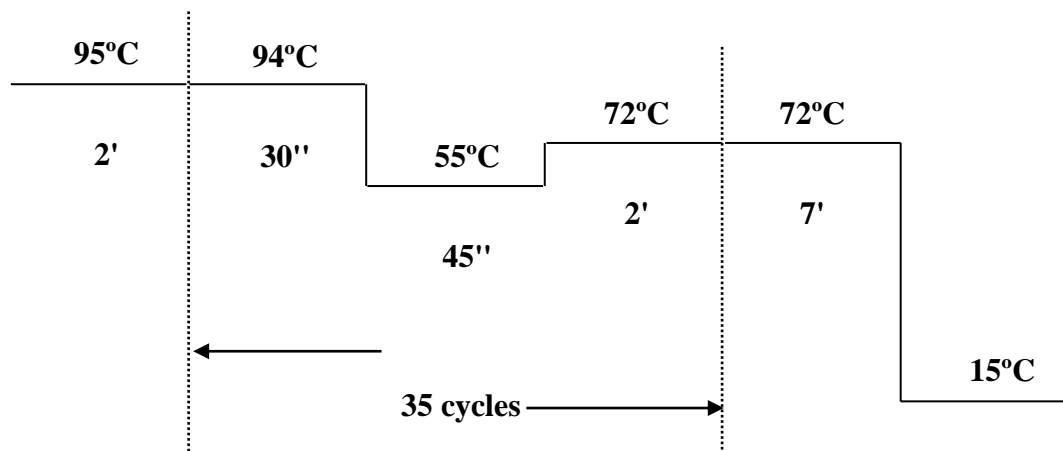
Figure 4.2: Reverse transcription polymerase chain reaction$^\infty$ cycling conditions.

The amplification of N, F, H and P genes using both long and short primers where ' represents minutes, " the seconds, ∞ infinity and oC Celsius degree.

4.2.4.5 Gel electrophoresis and visualization

The electrophoresis on an agarose gel 1.5% in 0.5% TAE buffer stained with GelRed was used to separate the reverse transcription polymerase chain reaction products. Each well was loaded with 5 µl of the PCR product and 1 µl of 6X DNA loading dye orange and blue (Madison, USA). Samples were separated along with a 1000 bp plus DNA ladder/1kb plus ladder (Promega, Madison, USA) at 100 volts for 30 minutes. The agarose gel was visualized by ultraviolet fluorescence light using a transilluminator (Sigma-Alderich, St. Louis, USA).

4.2.4.6 Sequencing

Samples that tested positive for PPR virus using the longer N, F and H gene primers set together with the Nigeria vaccine (75/1), used as a positive control, were sequenced. However, those samples that tested positive with P gene primers set and all other short primers (NP3-NP4, F, H, qPCR and UP) were only run on Gel electrophoresis. Before sequencing directly using Big Dye Terminator Cycle Sequencing kit v3.1, fragments (PCR products) were purified with the kit of QIAquick Gel Extraction using manufacturer's protocol as presented in **Appendix 9**. Final concentration of the PCR product of each sample was checked with Nanodrop2000 and normalized at 30-50 ng/µl before sequencing. Subsequently PCR products with very low DNA concentration were purified in spin column using QIAquick PCR Purification Kit (QIAGEN, Cat No/ID: 28106) following manufacturer's instructions as described in **Appendix 10**. Sequencing of the amplified and purified N, F and H PPRV fragments was done by Inqaba Biotechnical Industries (Pty) Ltd in Pretoria, South Africa and Bioneer innovation Ltd in China following the manufacturer's instructions (**Appendix 11**).

4.2.7 Peste des petits rumiants virus isolation

Approximatively 20 samples which were positive by the competitive Enzyme Linked Immunosorbent Assay (cELISA) were processed and cultured in VERO cells (African Green Monkey Kidney cells) to obtain virus isolates following the instruction as suggested by Ozkul *et al.* (2002) (**Appendix 12**).

4.2.8 Data analysis

Sequences were trimmed and assembled (both forward and reverse). The forward and reverse complement nucleotide sequences delimited by forward and reverse primers of several N, F and H gene PCR products of PPRV from the same location were aligned using CLC workbench 7.7.2 (Qiagen, Hilden, Germany) to obtain a consensus nucleotide sequence. One consensus sequence from each of the three sequenced genes (N, F and H) was used to search for other highly similar sequences (mega blast) available in the GenBank (NCBI) using the BLAST online tool. Highly similar sequences (from 97-100% identity and 99-100% query cover with high score and low E-value ≤0) from different African and Asian countries representing all the four PPRV lineages were downloaded and saved in FASTA format with their access numbers which were later included in the phylogenetic trees.   These downloaded sequences were then selected based on the criteria of the information available regarding the location and the year of isolation. Similarly sequences of Rinderpest (NC_006296.2), Canine distemper (NC_001921.1) and Measles (NC_001498.1) viruses were used as outgroup controls. Using Clustal-W algorithm incorporated in MEGA v6.0 as suggested by Kumar *et al*. (2016), the retrieved data were aligned separately and phylogenetic analyses were carried out using the neighbour joining method following the Kimura 2-parameter nucleotide substitution model with 1000 bootstrap replicates to test the robustness of the tree topology (Tamura *et al.,* 2011). The prevalence was calculated using a proportion formula:

$$P = \frac{n}{N} \, 100$$

Where, **p** is a prevalence (using RT-PCR), **n** is the total number of positive samples and **N** the overall number of tested samples.

4.3 RESULTS

4.3.1. RNA quality and quantity

The total RNA that was obtained after extraction was intact because the 28S and 18S sharp and rRNA bands (eukaryotic samples) appeared after running on a denaturing gel, (**Figure 4.3**). The 28S rRNA band was almost twice as intense as the 18S rRNA band. This 2:1 ratio represented by (28S:18S) found is a good indication that the RNA was integral/intact. However, a smear appearance would have been seen if the extracted RNA were partially degraded, with lack of the sharp rRNA bands, or would not exhibit a 2:1 ratio. Moreover, extracted RNA which is completely degraded would appear as a very low molecular weight smear. The quality and quantity of the RNA obtained using Bionneer and hereby presented as RIN^e value for each individual sample below the gel image (**Figure 4.4B**) showed a representative electropherogram of total RNA from goat samples. The 28S and 18S peaks are annotated for easy interpretation (**Figure 4.4A** and **Figure 4.5).** RNA concentrations of the 150 tested samples were good and varied from 10.2 ng/µl (sample number 228) to 634.8ng/µl (sample 44) (**Table 4.3**).
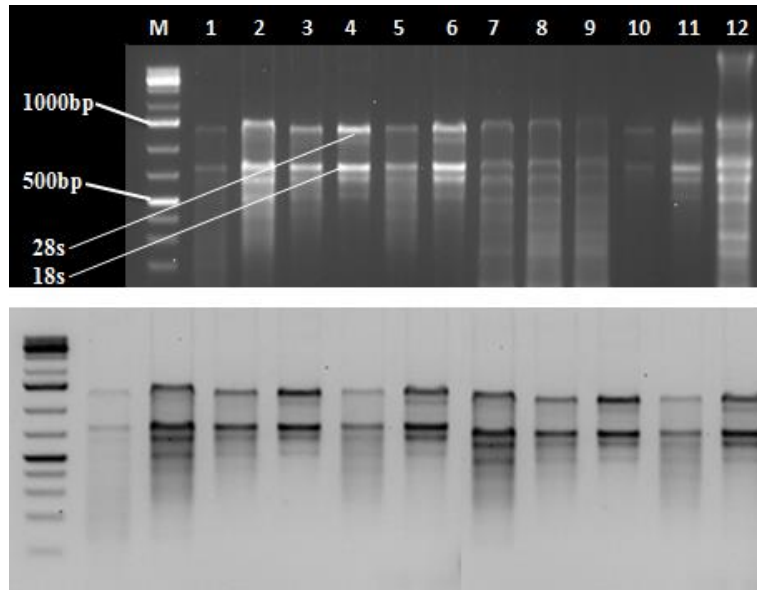
Figure 4.3: Gel electrophoresis RNA image (1.2%, 7 volts/cm, 0.5X TBE, 0.1%DEPC). RNA products visualized under UV transilluminator showing the RNA integrity (the presence of the 28s and 18s RNA) where M is the DNA marker (1kb plus), 1-12 RNA samples.
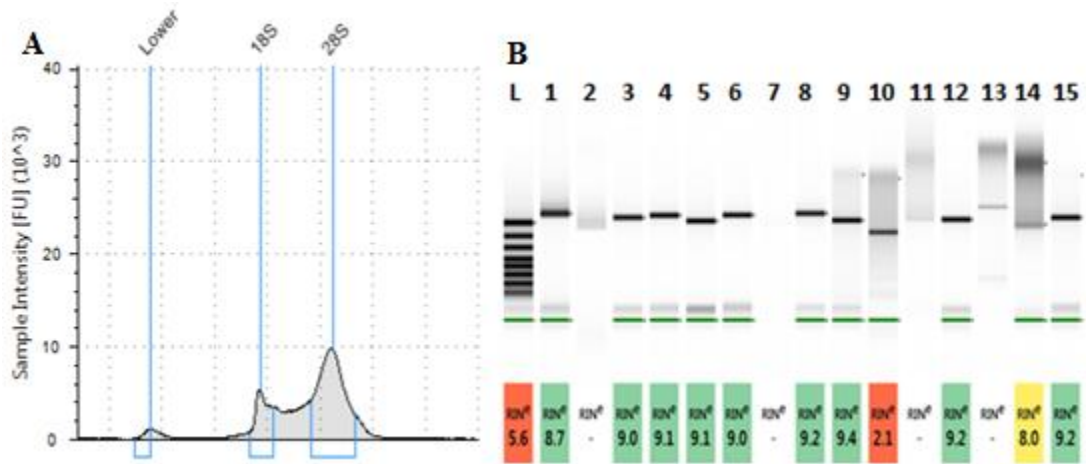
Figure 4.4: RNA analysis carried out using the Agilent 2200 TapeStation system.

(A) Representative electropherogram of total RNA, the 18/28S peaks are annotated; **(B) Gel image showing different RNA samples from goats and sheep.**
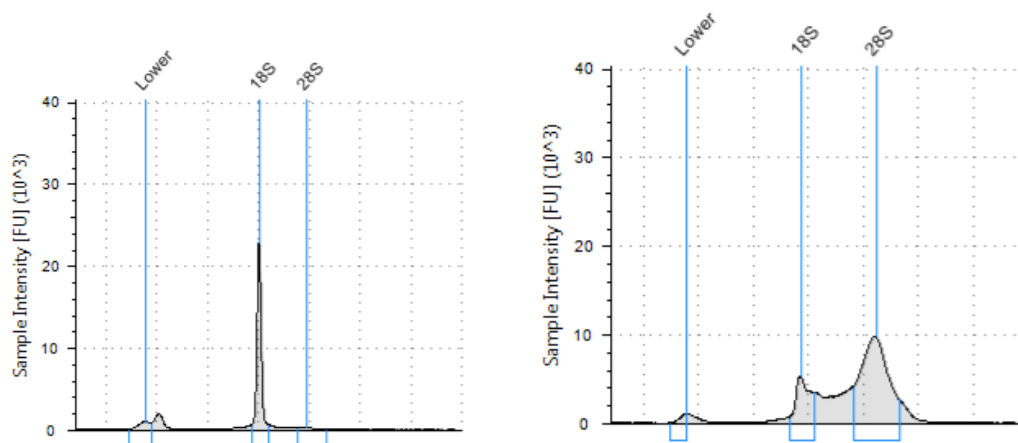
Figure 4.5: Electropherogram of total RNA from goat and sheep samples from South Kivu, the 18/28S peaks are annotated.

The minimum RNA concentration obtained was 10, 2 ng/µl, while the maximum was 634, 8 ng/µl. To assess the RNA and DNA purity with reference to RNA absorbance at 260 nm, the ratio of absorbance at 260 nm and 280 nm was good for many samples from South Kivu (~2.0 which is normally recognized as "pure" for RNA), but for some other samples this ratio was lower which could indicate either the protein or phenol contaminants that absorb strongly at or/ near 280 nm (**Table 4.3** and **Figure 4.6 A and B**). Similarly, the values of the 260/230 ratio, used as a secondary measure the purity of nucleic acid, were higher (2.0-2.2) compared to the respective values of 260/280 ratio for many samples (**Appendix 13**). However, the lower values of this 260/230 ratio

obtained from same samples could be due to the occurrence of impurities which normally absorb at 230 nm (**Figure 4.6 A a**nd **B**). In all the cases all the samples were amplified for PPR diagnostic.

Table 4.3: Total RNA concentrations and purity using NanoDrop® ND-1000

Sample ID	NA (ng/µl)	A260	A280	260/280	260/230	Factor
Maximum	634.8	15.87	7.388	2.15	0.47	40
Minimum	10.2	0.256	0.114	2.24	0.1	40

NA: Nucleic Acid concentration

(A)

(B)

22

252.6ng/µl

260/280:2.08

260/230:2.20
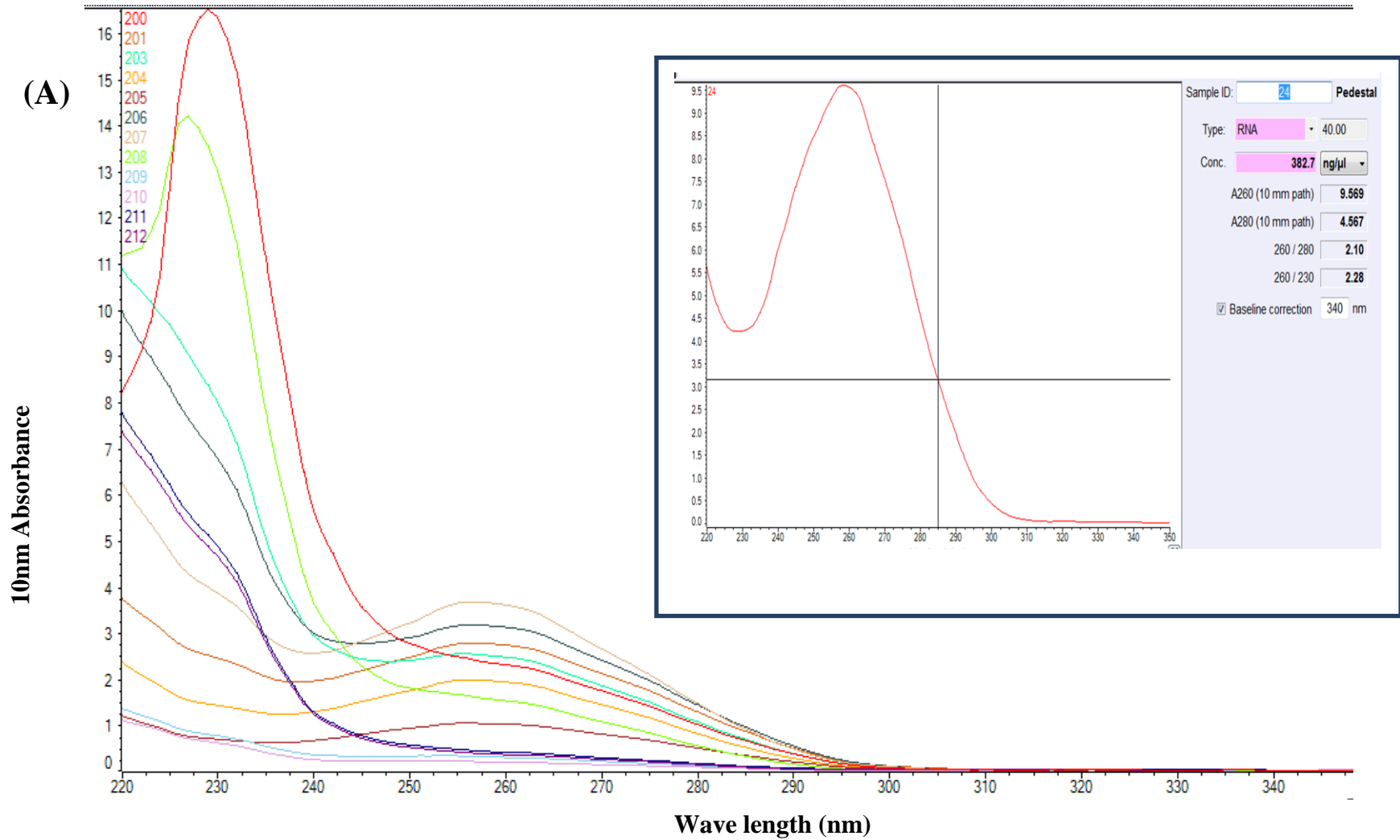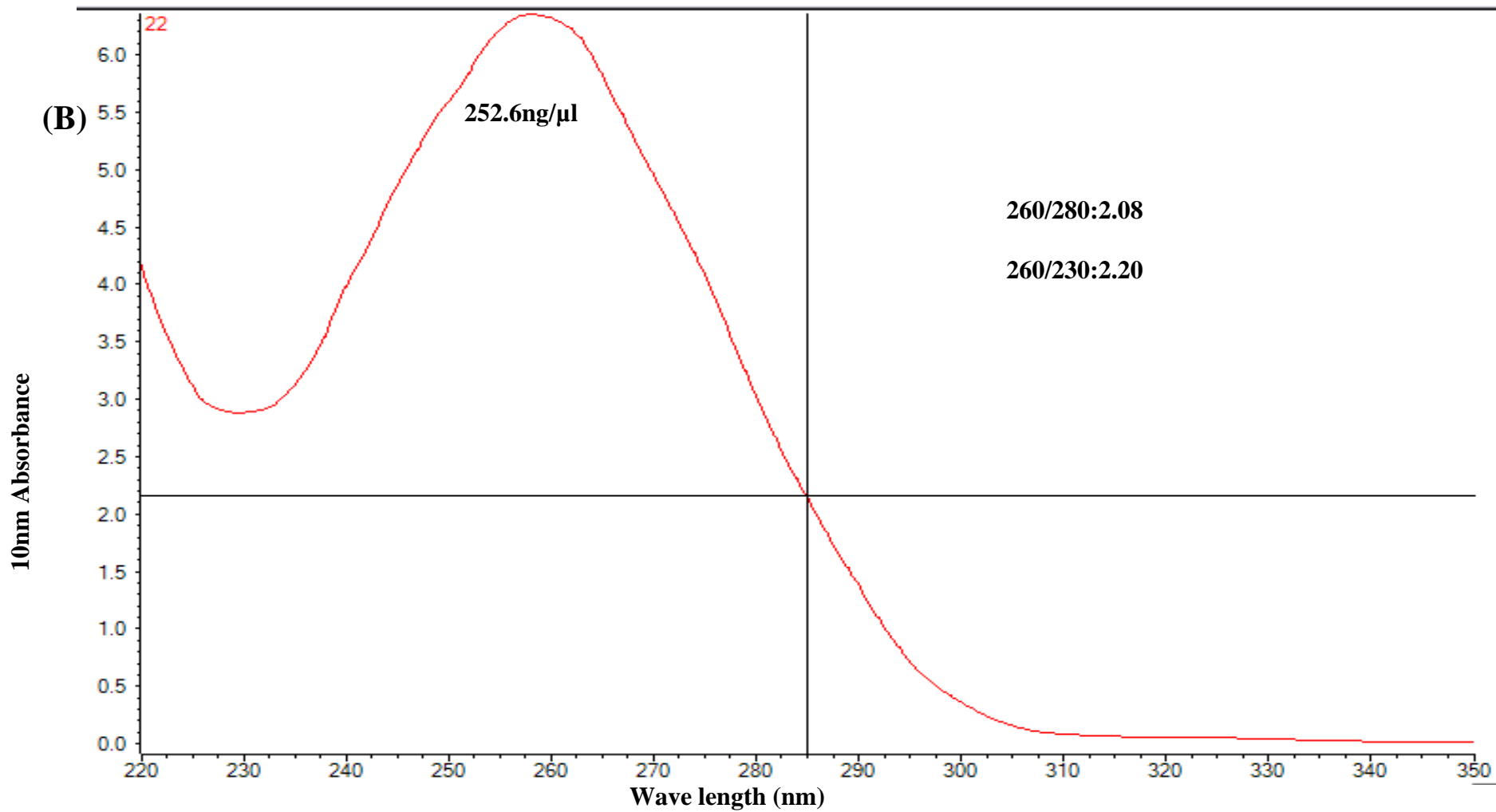
Figure 4.6: Typical spectral pattern for RNA samples using a NanoDrop® ND-1000.

4.3.2 Detection of PPRV by RT-PCR

PPRV positive samples were detected using gene-specific designed and published primers targeting fusion (F) (**Figure 4.7**), nucleoprotein (NP) (**Figure 4.8**) and hemagglutinin (H) genes (**Figure 4.9**) with both long and short amplicons according the primers used.

4.3.2.1 PPRV Fusion gene

Fusion gene amplicons of 1376 (**Figure 4.7A**) and 372 (**Figure 4.7B**) base pairs were amplified.
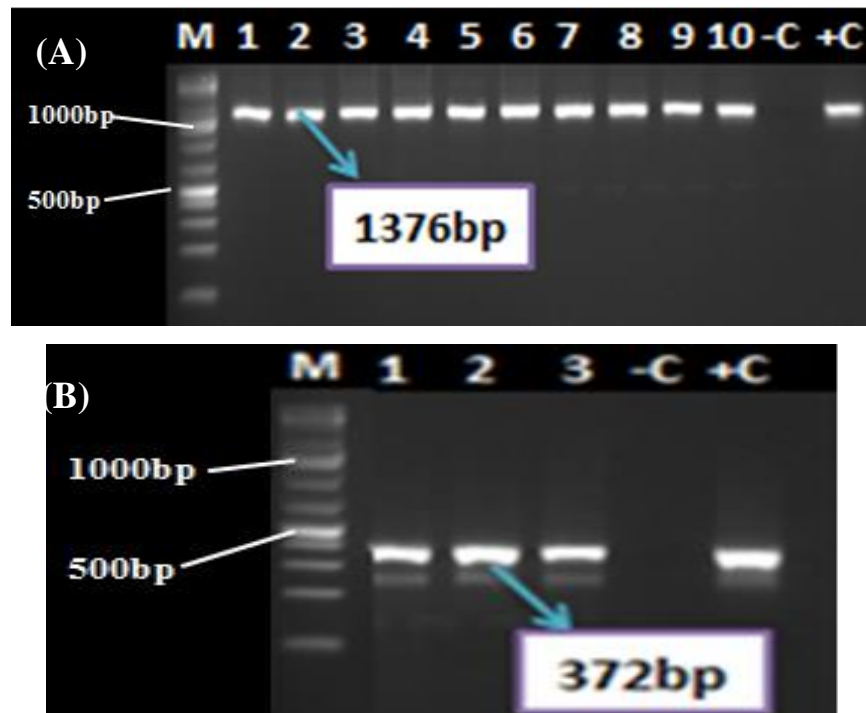
Figure 4.7: RT-PCR products visualized under UV transilluminator showing fusion gene amplicons. **(A) Represent a long amplicon obtained using the FL set of primers and (B) represent the short amplicons obtained using F sets of primers. M is the DNA marker (1kb plus), 1-10 samples, -C is negative control and +C is a positive control.**

4.3.2.2 PPRV Nucleoprotein gene

PPRV nucleoprotein amplicons of 1430 (**Figure 4.8A**) and 350 (**Figure 4.7B**) base

pairs were amplified.
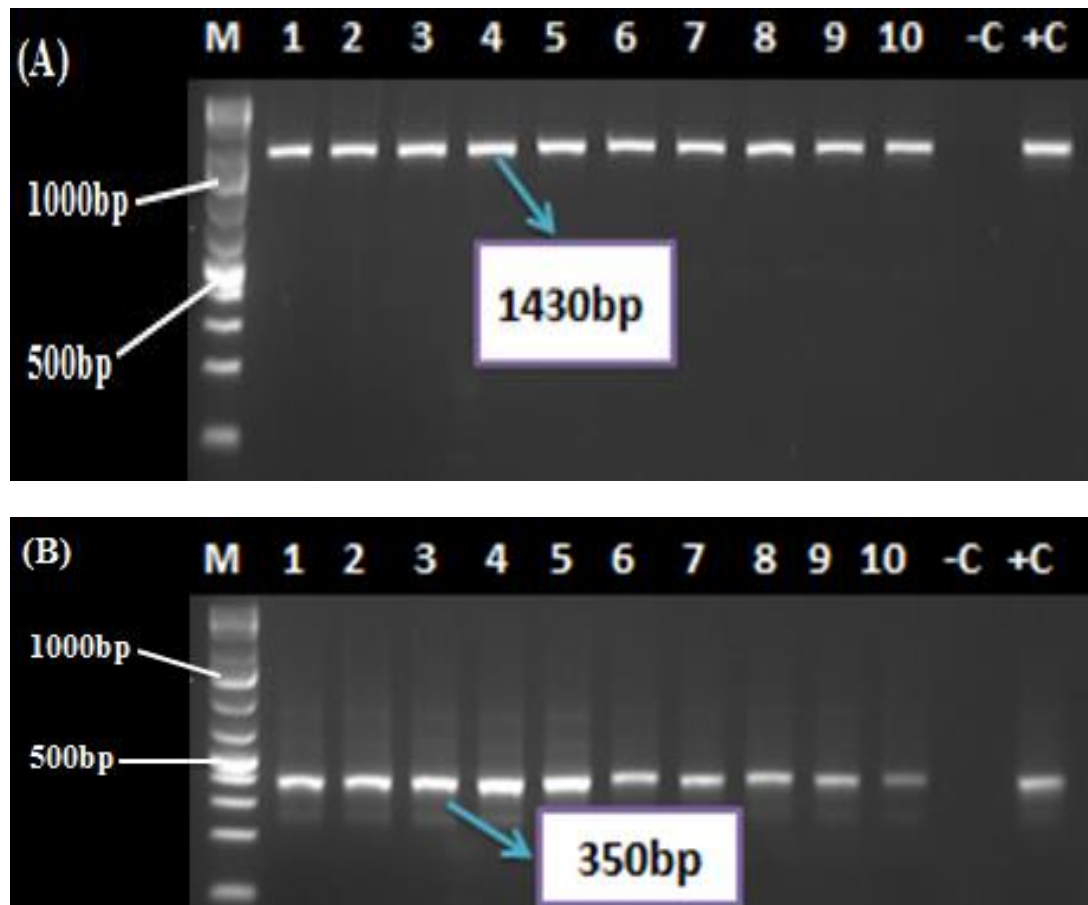
Figure 4.8: RT-PCR products visualized under UV transilluminator showing nucleoprotein amplicons. (A) Represent a long amplicon obtained using the NL set of primers and (B) represent the short amplicons obtained using NP3-NP3 sets of primers. M is the DNA marker (1kb plus), 1-10 samples, -C is negative control and +C is a positive control.

4.3.2.3 PPRV Hemagglutinin gene

Amplicons of 1489 (**Figure 4.9A**) and 410(**Figure 4.9B**) base pairs were amplified in hemagglutinin gene.
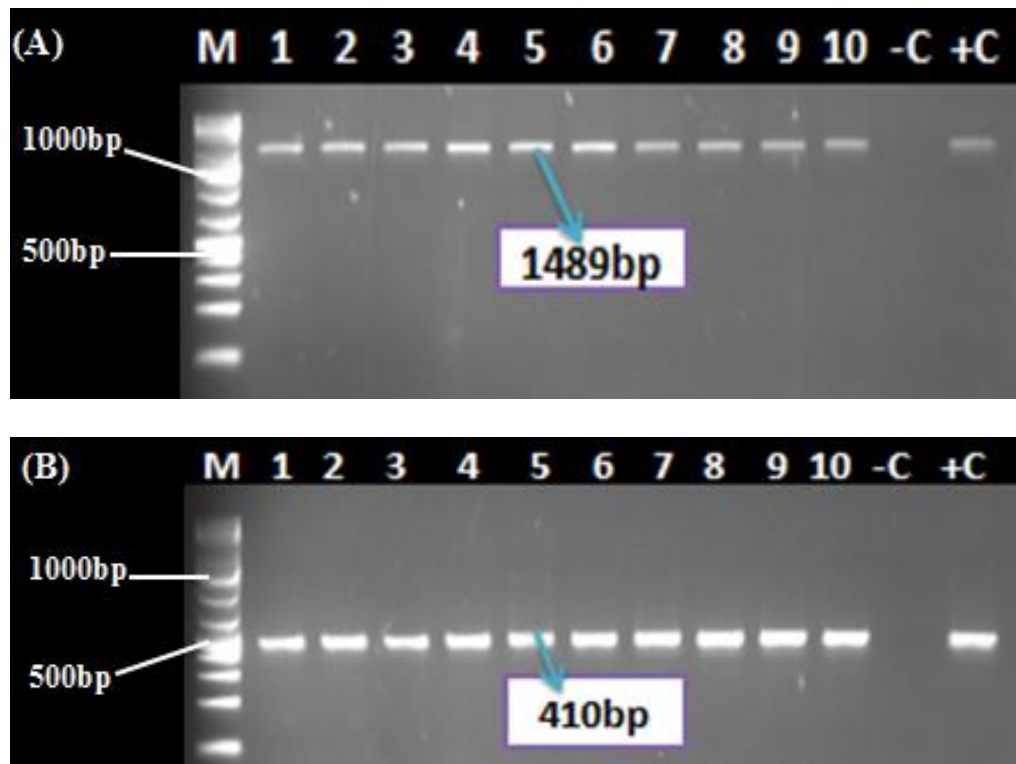
Figure 4.9: RT-PCR products visualized under UV transilluminator showing hemagglutinin gene amplicons.(A) Represent a long amplicon obtained using the HL set of primers and (B) represent the short amplicons obtained using H sets of primers. M is the DNA marker (1kb plus), 1-10 samples, -C is negative control and +C is a positive control.

4.3.3 Determination of PPRV prevalence using RT-PCR

Out of 150 animals (120 goats and 30 sheep) sampled, 97 were positive (64.7%, n=150) and 53 (35.3%) were negative using both three PPRV genes (nucleoprotein, fusion protein and hemagglutinin). The prevalence was high in Shabunda (87.5%), followed by Mwenga (77.1%), then Fizi (70%). No positive animal was found in Kalehe (**Table 4.4**).

Table 4.4: RT-PCR-based PPRV prevalence and distribution in South Kivu.

Location	Sample type	Positive	Negative	Total	Positivity (%)
Mwenga	Swabs	9	1	10	90
	Whole blood	13	7	20	65
	Tissues	5	0	5	100
	Total	**27**	**8**	**35**	**77.14**
Shabunda	Swabs	14	0	14	100
	Whole blood	15	5	20	75
	Tissues	6	0	6	100
	Total	**35**	**5**	**40**	**87.5**
Fizi	Swabs	14	2	16	87.5
	Whole blood	10	10	20	50
	Tissues	4	0	4	100
	Total	**28**	**12**	**40**	**70**
Kalehe	Swabs	0	13	13	0.0
	Whole blood	0	20	20	0.0
	Tissues	0	2	2	0.0
	Total	**0**	**35**	**35**	**0.0**
South Kivu	**Total**	**97**	**53**	**150**	**64.7**

4.3.4 Phylogenetic analysis of PPRV

4.3.4.1 Phylogeny of PPRV Fusion gene

The multiple alignment of F gene sequences showed some mutation where Adenine (A) was replaced by Thymine (T) at 346th, 371th, 374th base pairs; cytokine (C) by Guanine (G) at 378th base pairs and Thymine (T) to Adenine (A) at 392th position for a small fragment of 49 base pairs as showed in circle (**Figure 4.10**). Several more mutations appeared in several parts of the whole amplified amplicon.
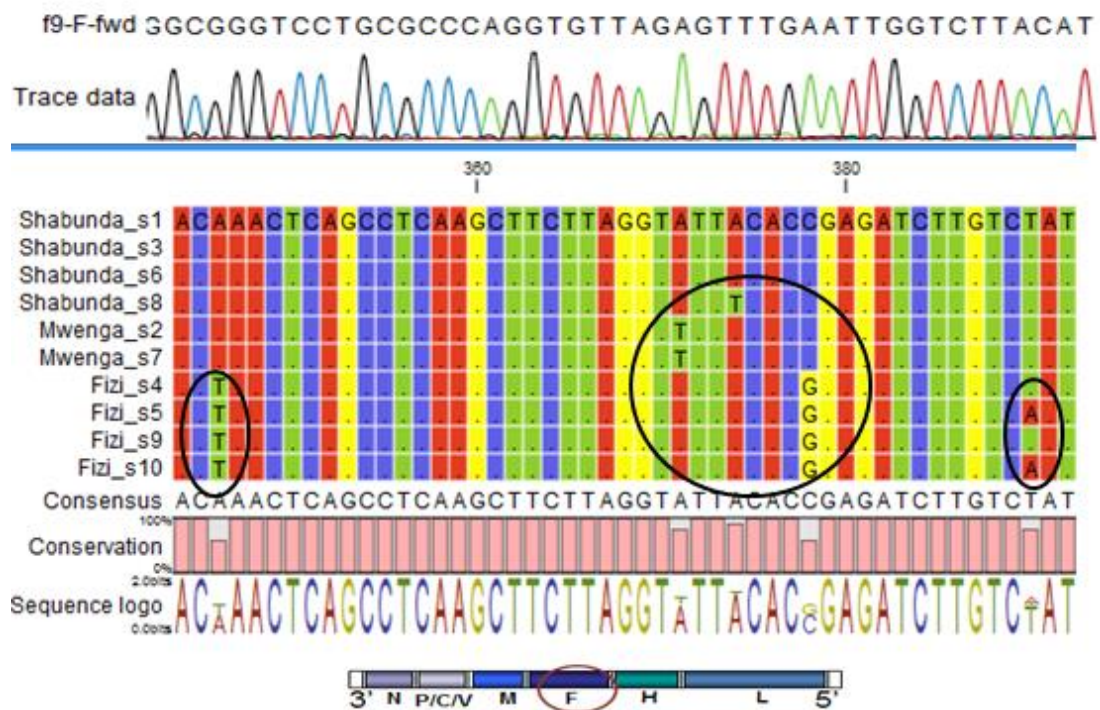
Figure 4.10: Multiple alignment of small fragment of the F gene amplicon (from 344th to 394th base pairs).

Phylogenetic trees based on F gene nucleotide sequences obtained in this study clustered the PPRV from South Kivu (Shabunda, Mwenga and Fizi) indicated by a

black dot in lineage III together with other PPRV previously reported from Ethiopia, Tanzania Uganda and Kenya (**Figure 4.11**).
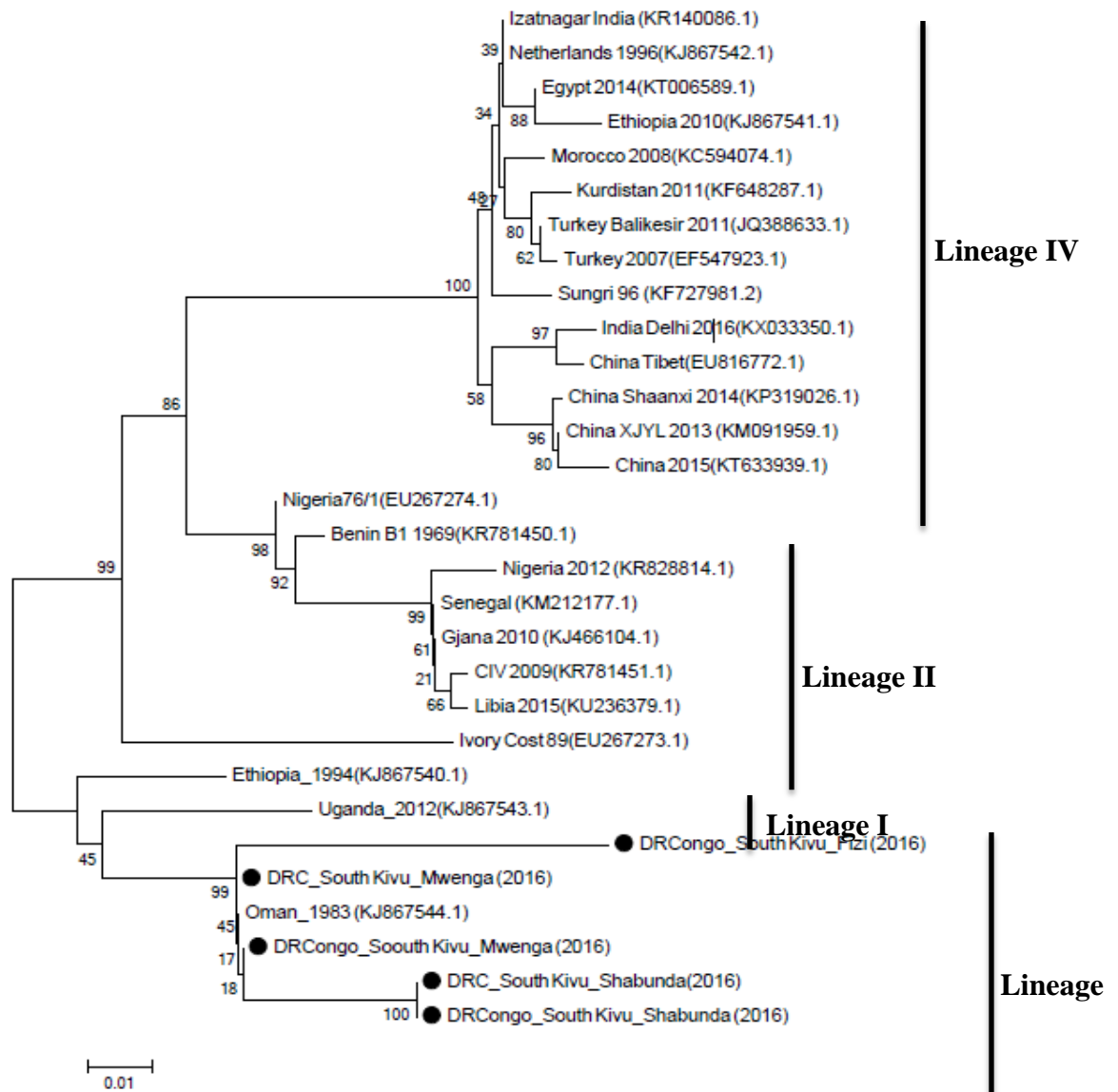
Figure 4.11: Neighbour-joining tree based on PPRV F gene sequences. Where Scale bar using Kimura 2-parameter model with 1000 bootstrap replicates shown next to the branches presents the nucleotide exchanges per site. Samples from South Kivu are presented with a black dot.

4.3.4.2 Phylogeny of PPRV Nucleoprotein

Almost same mutations as described in section **4.3.3.1** occurred in the nucleoprotein gene. The samples from South Kivu are represented by black dots clustered with PPRV from lineage III (**Figure 4.12**).
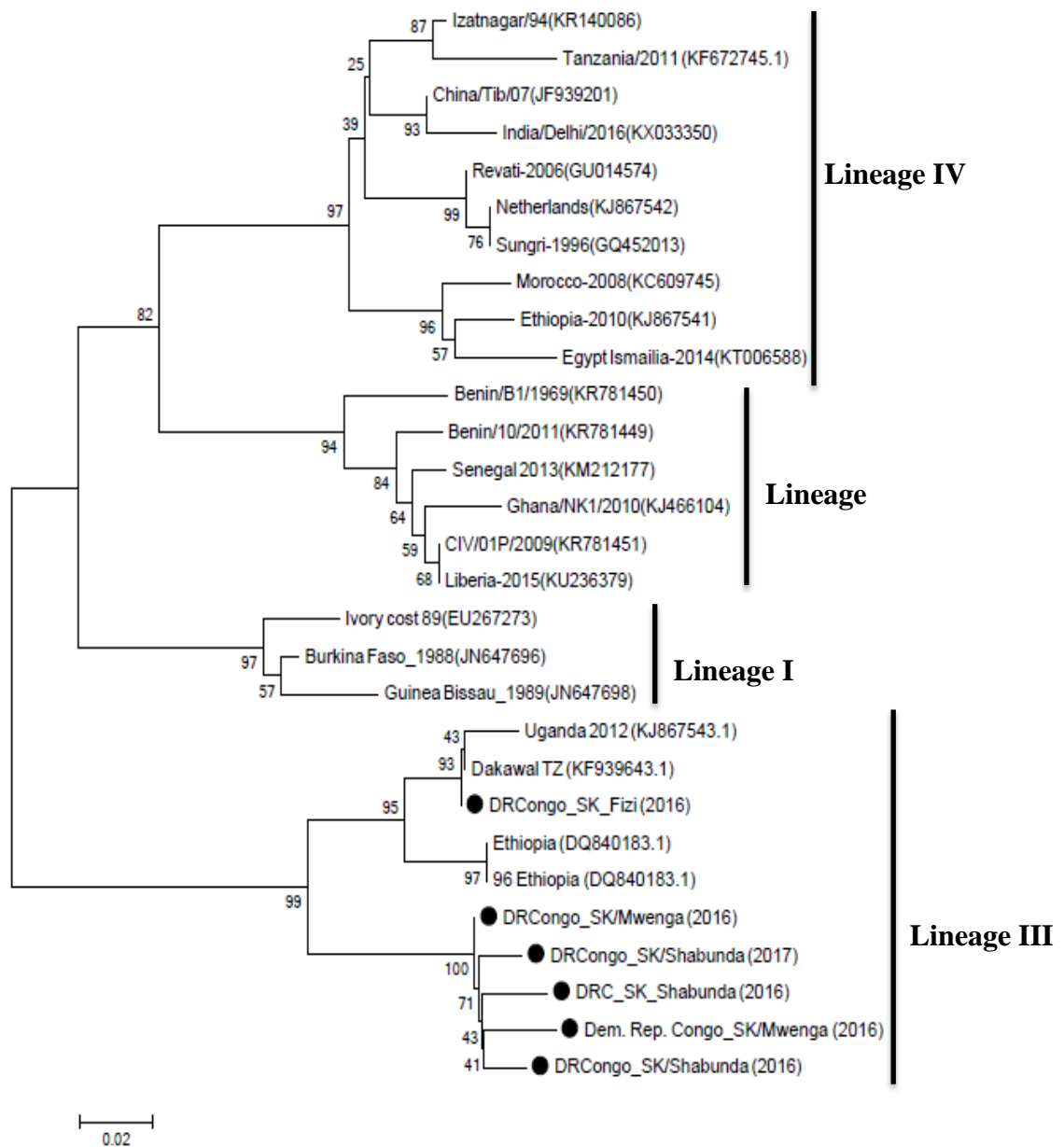
Figure 4.12: Neighbour-joining tree for PPRV N gene sequences.

4.3.4.3 Phylogeny of PPRV Hemagglutinin gene

Phylogenetic relationship of the hemagglutinin gene also indicated that the PPRV isolates were of lineage III (**Figure 4.13**). The samples from South Kivu are represented by black dots.
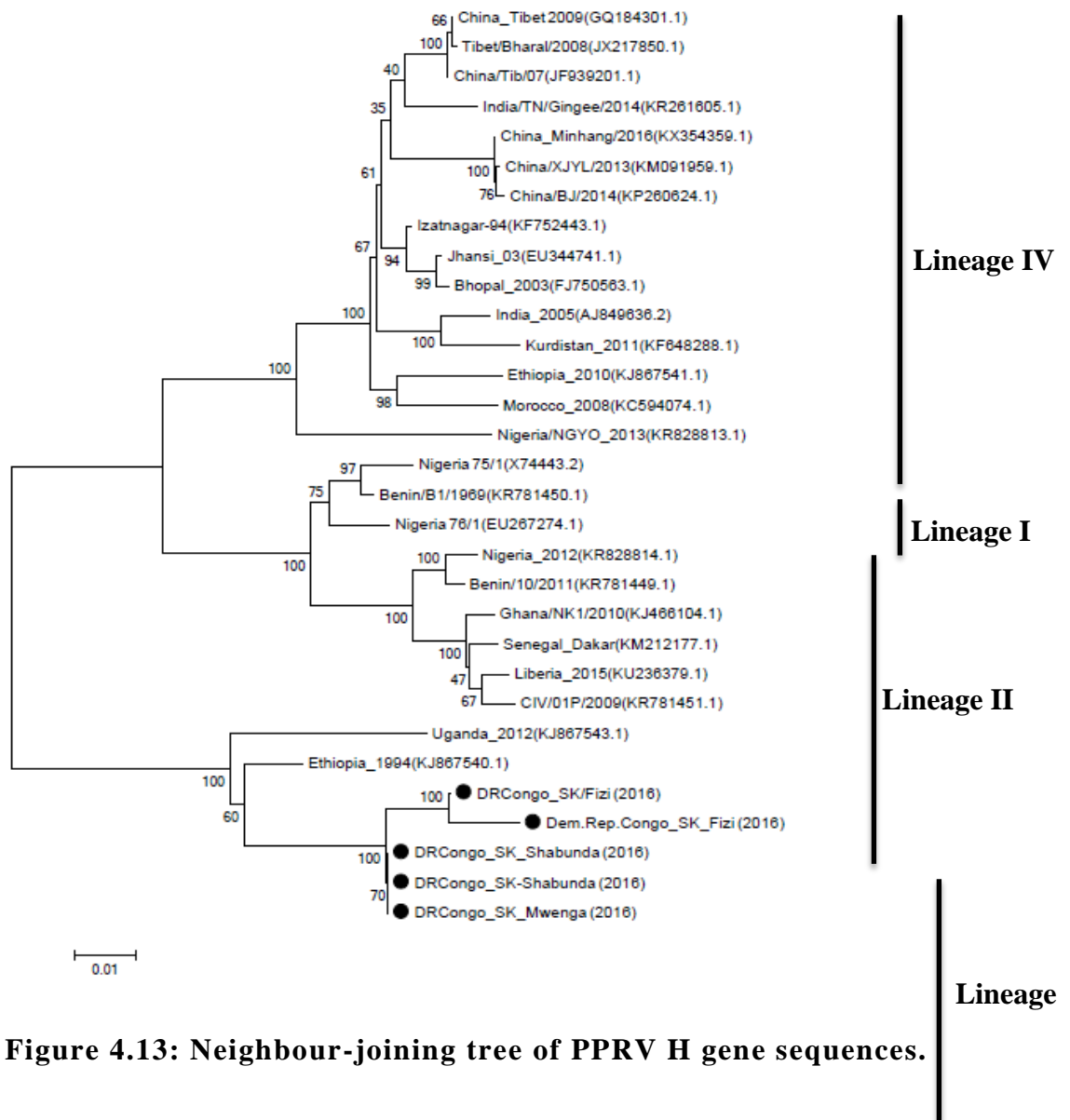
Figure 4.13: Neighbour-joining tree of PPRV H gene sequences.

4.3.5 Genetic comparison of PPRV-fusion protein and Nigeria 75 Vaccine

A multiple alignment of PPRV fusion gene sequences from South Kivu (1-10) compared to the Nigeria 75_vaccines used in the country showed a mean of 87% of identity or region of similarity in a fragment of 1376 bp (**Figure 14.A**). Over 10% of nucleotide sequences were constituted with regions of dissimilarity, mismatched with the Nigeria 75_vaccines sequenced (**Figure 4.14B**). The phylogeny supports this result when compared to other related morbilliviruses sequences (**Figure 4.15**).

Figure 4.14: Alignments of PPRV F gene sequences from South Kivu and Nigeria 75_vaccine. (A) Pairwise comparison of similarity; (B) Multiple alignments of sequences.

Figure 4.15: Phylogenetic tree of PPRV Fusion gene sequences, Nigeria75_vaccine and other morbilliviruses.

4.3.6 Isolation of PPR Virus

The isolation and identification of peste des petits rumiants virus from ocular and nasal discharges was done through cytopathic effects on veroSLAM Cell passage. The cytopathic effects (CPE) between the VERO Cells and PPR virus were observed on 2-4 days post-inoculation of the virus. Rounding of cells were observed during the initial stage of culture and later on clumping and elongation of VERO cells (**Figure 4.16**). The PPRV was harvested when 60-90% of the cells showed cytopathic effects.
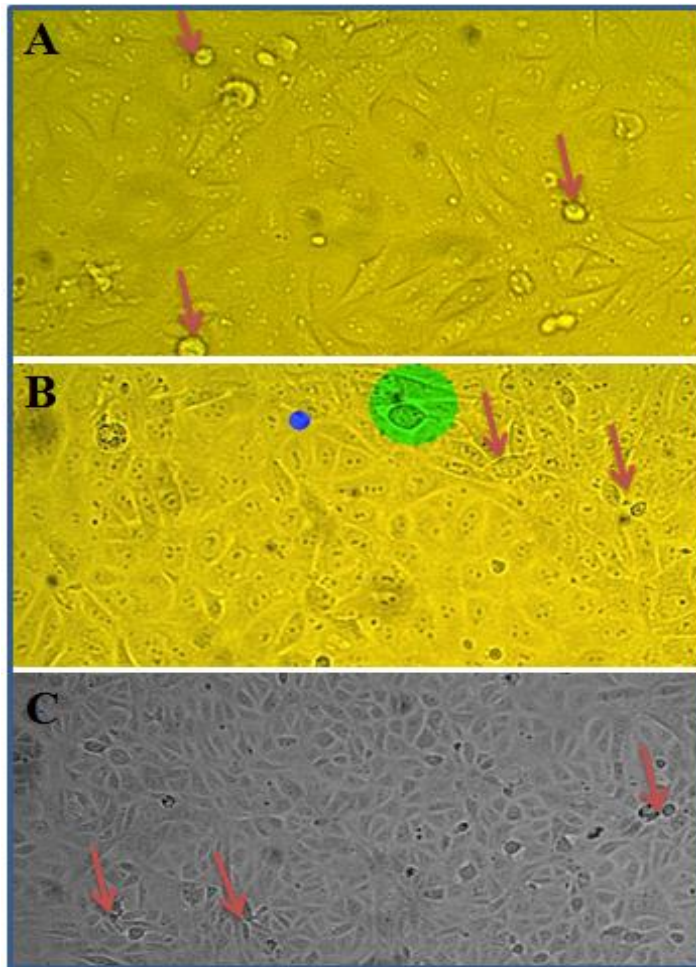
Figure 4.16: Cytopathic effects (CPE) on VERO cells due to PPRV. (A) CPE-Rounding of VERO cells, (B) CPE-Elongated VeroSLAM Cells, (C) CPE-Clumping of VeroSLAM Cells.

4.4 DISCUSSION

The circulation of PPRV in selected parts of Eastern D R Congo, in South Kivu province, including Shabunda, Mwenga, Fizi and Kalehe counties was investigated in the present study. The PPRV prevalence with RT-PCR in South Kivu was very high

(64.7%) and distributed widely in the region (**Table 4.4**). This prevalence was higher compared to the serological prevalence of 45.3% that was found using competitive Enzyme Linked Immunosorbent Assay, described in the chapter three, section 3.3.1. Although clinical outbreaks of PPR have been reported before in the South Kivu region (Veterinary Record, 2012 and SADC, 2012a and b), neither molecular nor serological diagnostic tools were used to confirm the presence of the disease in small ruminants in this region. Previous studies done in Tanzania have detected PPRV genome in the range of 29.6 - 31.1% in the goats tested (Kgotlele *et al.* 2014; Muse *et al.,* 2012 and Kivaria *et al.,* 2013).

Irrespective of the length of the gene fragments used in this study , all the phylogenetic trees constructed using the partial F (**Figure 4.11**), NP (**Figure 4.12**) or H (**Figure 4.13**) gene sequences clearly distinguished the four different lineages of PPRV compared with the strains of the PPRV lineage III indicated with black dot in all the phylogenetic trees constructed that were found in Eastern part of the Democratic Republic of Congo (Mwenga, Shabunda and Fizi counties) with high bootstrap support values (1000). Muniraju *et al.* (2014) confirmed a co-circulation in the Middle East and East Africa of PPRV lineage III and IV in their study of the origin of the complete PPRV genome of lineage III for comprehensive understanding of the molecular evolution and emergence of PPRV. Although, D.R. Congo was not included in the study assumptions, the lineage III of PPRV might be found also in Eastern part of the country because it was diagnosed in many neighboring countries in East but this could differ with the PPRV

strains in circulation in Western part of the country which shares borders with Central African countries. For instance, lineage III was found in Angola (OIE, 2012) and in D.R. Congo (Dundon *et al*, 2015 unpublished data), suggesting the need to determine whether the southward movement of lineage IV PPRV will displace the predominant lineage III viruses currently in East Africa. Dundon *et al.* (2015) found that the PPRV causing disease in Kenya was 95.7% identical to the full genome of a virus isolated in Uganda in 2012 and that a segment of the viral fusion gene was 100% identical to that of a virus circulating in Tanzania in 2013. Therefore, these data strongly show the transboundary movement of lineage III viruses between East African countries which has significant implications for surveillance and control of this important disease as it moves southwards in Africa. The results obtained also show that PPRV is circulating in goats in South Kivu as confirmed by molecular detection of PPRV genes. Furthermore, previous reports have confirmed the presence of PPR in Western DRC (Veterinary Record, 2012 and SADEC, 2012a and b) but the study did not characterize the strains that were circulating (No sequences available from DRC in the GenBank). Several studies also reported the circulation of PPRV of Lineage III in East Africa (Swai *et al*., 2009; Karimuribo *et al.,* 2011; Kivaria *et al*., 2013; Luka *et al*., 2012b; Lembo *et al.,* 2013; Kihu *et al*., 2015a).

The presence of lineage III of PPRV in South Kivu, being similar to lineages found in East Africa can be explained by the fact that PPR is a transboundary disease and there is uncontrolled animal movement within and across the porous borders. Previous study by Domenech *et al.* (2006), reported the trade of live animals at markets has an important

conduit for transmission of infectious diseases. Simirlary, the role of animal markets in the PPR transmission was also previously reported by Muse *et al.* (2012), who found that poor or inappropriate infrastructure such as local animal markets may be facilitating the PPR transmission.

The cytopathic effects including rounding, elongating and clumping of VeroSLAM cells produced by the PPR virus found in this study were similar to those produced by rinderpest virus on VERO cells (Gopilo, 2005). Ozkul *et al.* (2002) reported almost similar observations showing initial rounding of VERO cells and later on development of syncitia. Although the cytopathic effects on PPRV were not very high on VeroSLAM cells due to limited time of inoculation, further studies were recommended for the Neutralizing antibody effects.

In conclusion, from the available gene sequences, including the data generated in this study, only lineage III has been found circulating in Mwenga, Shabunda and Kalehe counties in the Democratic Republic of Congo and this might have been introduced through animal trade market from East Africa, including Tanzania, Uganda and Kenya. Therefore, to control the spread of PPR, proper regulatory and bio-security measures should be adhered. Further studies on the complete PPRV genome from DRC (south Kivu) were suggested for a deeper understanding of the evolution and epidemiology of the virus.

CHAPTER FIVE: SUSCEPTIBILITY OF SOUTH KIVU GOATS TO *PESTE DES PETITS RUMINANTS*: MITOCHONDRIAL DNA CONTROL REGION (*D-LOOP*) ANALYSIS

5.1 INTRODUCTION

In Democratic Republic of the Congo, there are more than 21 million goats out of more than 350 million of goats found in Africa (FAOSTAT, 2014). Previous studies have classified these goats on molecular basis into six lineages [A, B (B1, B2), C, D, F, G] of mitochondrial DNA (mtDNA) *d-loop* region which have phylogeographic structures that are weak according to Chen *et al*. (2005) and Naderi *et al.* (2007). Indigenous goats (*Capra hircus*) are adapted better and can survive under the harsh environmental conditions when compared with their exotic counterparts (Jimmy *et al.,* 2010). These indigenous goats are also tolerance/resistance to diseases and pests, have high fertility with good maternal qualities (Bruford and Wayne, 1993; Wollny, 2003; Naderi *et al.,* 2007).

The polymorphism of mitochondrial DNA (mtDNA), especially the displacement loop (*d-loop*) region, has been mostly useful to comprehend phylogenetical relationships in several animal species, including cattle (Mannen *et al.,* 2004), pig (Giuffra *et al.,* 2000), sheep (Hiendleder *et al.,* 2002), chicken (Liu *et al.,* 2004) and goat (Luikart *et al.,* 2001; Mannen *et al*., 2001; Sultana *et al.,* 2003; Chen *et al.,* 2005). Based on former reports, Capote *et al.* (2004) and Tarekegn *et al.* (2016) summarized the global distribution and routes of divergence of indigenous goats. *Peste-des-petits-ruminant* outbreaks were

severally confirmed in many neighboring countries to D R Congo such as Tanzania as reported by Kgotlele *et al.* (2014), Uganda as confirmed by Bonny *et al. (*2011) and Kenya, found by Simon *et al.* (2015). Kibegwa *et al.* (2016) in their study on goat's mt DNA genetic variability in Narok and Isiolo counties of Kenya showed that the genetic erosion reduced the adaptive potential of any species. Okomo-Adhiambo *et al.* (2002) emphasized on lack of information on the genetic variation amount present in African Indigenous breeds. This could limit the selection and conservation of these animals' resources. The DNA based techniques for goat breeds genetic characterization is more reliable because it is established on accurate genotypic information. The objective of this current research was, therefore, to examine the *d-loop* genetic diversity of mitochondrial DNA and establish the possible maternal lineages of indigenous goat breeds susceptible to *PPR* in South Kivu province in Democratic Republic of Congo.

5.2 MATERIALS AND METHODS

5.2.1 Sample collection and DNA extraction

The same 150 samples including tissue and blood collected as mentioned in Chapters four (as described in chapter 4, section 4.2.2**)** from farmers' flocks in three different agro-ecological areas including Mwenga-Shabunda, Fizi and Kalehe regions in South Kivu province were used to extract respective DNAs used for this study. The study involved three indigenous uncharacterized goat populations of Democratic Republic of Congo which were confirmed previously with PPRV using both serological (cELISA) assay (described in chapter three) and molecular (RT-PCR) tests presented in chapter four. The tissue and whole blood were processed and a total of 150 genomic DNA

samples were extracted using both phenol/chloroform protocol of Sambrook *et al*. (1989), Qiagen PurGene whole blood procedure and DNeasy extraction kit (Qiagen, Hilden, Germany, Model: 69506) following manufacturer's procedure (**Appendix 14**). Tissue and swab samples were ground and homogenized. Similarly, viral DNA was extracted from frozen blood samples using salt extraction method where one ml of blood was mixed with 4 mls of lysis buffer (Sucrose:109.536g, 1M Tris-HCL(P^H 7.5):10 mls, 1M MgCl2: 5ml and Triton-X 100 (Add last): 10 mls) to suspend the white pellet (**Appendix 15**). The quality and quantity checking of the viral DNA was as was done for extracted viral RNA (described in chapter 4, Section 4.2.4.2).

5.2.2 PCR amplification of *D-loop* region

Conventional PCR amplification of mtDNA *d-loop* region was carried out. A pair of primers forward: 5'-GAAGCCATAGCCTCACTATC-3 and reverse primer: 5'-GTTGGTACACTCATCTAGGC-3') to amplify a 1,220 bp mtDNA *d-loop* amplicon. Amplifications by polymerase chain reaction (PCR) were done using AccuPower® PCR Premix to which 0.2 µM of each primer was added, 1.5% Hi-DiTM formamide, 0.005mg of Bovine Serum Albumin (BSA) and 50 ng of DNA template to get a final reaction volume of 20 µl.

One stage touch-down PCR cycling profile was used for PCR amplification. It involved an initial denaturation step at 95^oC for 1 minute that was followed by 35cycles: a denaturation stage for 30 seconds at 95° C, annealing for 30 seconds at 57° C and 60 seconds for extension at 72° C.

A final extension stage for ten minutes at 72° C was done to finish the PCR amplication. The GIAGEN (QIAquick® PCR Purification) kit was used purified PCR products following manufacture's instruction (**Appendix 16**) before sequencing.

5.2.3 Sequencing of *d-loop*

The sequence of PCR products purified was done using the BigDye Terminator v3.1 Cycle Sequencing Chemistry and the ABI Prism 3130XL automatic capillary sequencer following manufacturer's recommendations (**Appendix 11**).

5.2.4. Data analysis

After sequencing, the generated chromatograms were visualized with the CLC workbench v7.0.4. All the amplified mtDNA *d-loop* region sequences belonging to goats from Mwenga-Shabunda, Fizi and Kalehe regions of South Kivu in Democratic Republic of Congo were used in this analysis. The *C.hircus* reference sequence from the GenBank with accession number GU223571 (*direct submission*) was saved and used search for variable sites scored/called against the *C.hircus* reference. To facilitate the recognition of haplogroup status of each individual, 22 goat mtDNA Hypervariable I region reference sequences (HV1: 481bp) belonging to the six known haplogroups/ lineages that were recommended by Naderi *et al.* (2007) were also downloaded from GenBank and included in this analysis (as given in Chapter two, Section 2.16, **Table 2.1**). Multiple sequence alignments were done in CLC with ClustalW algorithm and edited manually in MEGA 6 as suggested by Tamura *et al.* (2013).

The DNA Sequence Polymorphism (DnaSP) package v5 10.01 was used to produced and collapsed all the amplified sequences as suggested previously by Librado and Rozas (2009). The genetic diversity symbolized as the haplotypes number, haplotype diversity (h) with its standards error (SE), nucleotide diversity (π) and its standards error (SE). Arlequin 3.0 software was used for population determination by calculating the mean number of nucleotide differences between haplotypes. The neighbour-Joining (NJ) algorithm with 1000 bootstrap value using Kimura 2-parameter model as implemented in MEGA6 to visualize the genetic relationship between individuals and populations was used for phylogenetic tree construction for all the haplotypes.

The Network v4.6 software (http://www.fluxus-engineering.com/sharenet.htm) was used to construct the median-joining (MJ) network to complement the NJ tree in order to obtain further insights and in greater detail, into the genetic relationships between the haplotypes. To determine whether patterns of mitochondrial sequence variation were consistent with predictions of the neutral model and to infer the demographic history of these three goat breeds as suggested by Santos *et al*. (2010), D and Fu's Fs tests were estimated.

The sum of squares deviation (SSD) of the goodness of fit statistic and the raggedness index as suggested by Harpending (1994) were used to test the goodness of fit of the observed pattern to that projected under a demographic equilibrium. The analysis of molecular variation (AMOVA) was performed using Arlequin to assess the partitioning of genetic diversity and variation amongst populations and groups of populations.

5.3 RESULTS

5.3.1 Mitochondrial DNA *d-loop* amplification

Out of a total of 150 DNA samples extracted from goat samples only 111 samples amplified with amplicon of 1, 220 bp long with good tight white bands (**Figure 5.1**). These were now the samples that were sequenced for further analysis.
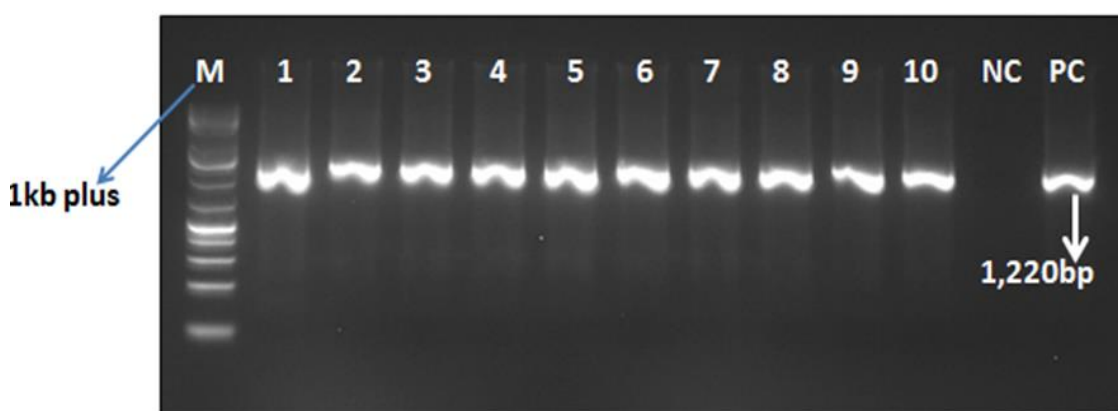
5.3.2 Mitochondrial DNA sequence and genetic diversity

Out of 111 *d-loop* mtDNA sequences analysed, 120 polymorphic/variable sites out of which 40 were for singleton variable sites and 80 for parsimony informative sites in a 1,220-bp sequence and 56 haplotypes were observed. All the three goat populations were therefore defined by a high level of genetic diversity. The total mutations number recorded was 124. The haplotype diversity was very high in the studied goats with a value above 0.971 (**Table 5.1**). In one hand, the goat population of Shabunda-Mwenga and Kalehe presented the highest haplotype diversity level (Hd = 0.961 ± 0.010) and (Hd=0.974 ± 0.019) respectively. One the other hand, the lowest level was detected in Fizi goats (Hd = 0.873 ± 0.051).

Similarly, the average nucleotide diversity in the studied population was $0.01068 \pm$ 0.00206 and ranged from 0.00573 ± 0.00137 in Fizi population to 0.00976 ± 0.00155 in Shabunda-Mwenga population which is closer to Kalehe goats ($\pi = 0.00573\pm 0.00137$) (**Table 5.1**). The average number of nucleotide differences was 10.7 ranging from 6.75 for Fizi to 11.7 for Shabunda-Mwenga goats.

Table 5.1: Mitochondrial DNA *d-loop* haplotypes diversity of DRC goats

Beed/Goat's Population	N	S	Eta	H	d±SD	π ±SD	k
Shabunda-Mwenga	55	72	72	27	0.961 ± 0.010	0.00976 ± 0.00155	11.7
Kalehe	22	65	66	16	0.97 ± 0.019	0.00899± 0.0018	10.7
Fizi	2	42	42	16	0.873 ± 0.051	0.073 0.00137	6.75
Overall	**109**	**120**	**124**	**56**	**0.971 ± 0.007**	**0.01068 ± 0.00206**	**10.7**

Key: N= Number of Individuals; **S**=segregating sites; **Eta**= No. of mutation; **Hd**= Haplotype diversity; **H**= No. of Haplotypes; **SD**=standard deviation; **π** =Nucleotide diversity and **k**=Nucleotide differences average number; DRC – Democratic republic of Congo.

5.3.3 Population phylogenetic analysis

To construct the phylogenetic relationship using neighboring joining algorithm and phylogenetic network, only the HVI region was used. The DRC goats were aligned together with 22 reference sequences which represent the five globally identified haplogroups. The analysis showed that the goats from D R Congo were divided into two

different mtDNA lineages (A and B) as presented in **Figure 5.2** and **Figure 5.3**. Lineage A had a total of 110 individuals (99.1%) and only one individual from Fizi goat population (0.9%) was aligned to lineage B. The Median-joining (MJ) network tree revealed the weak clusters to any population. All the goat populations from Shabunda-Mwenga and Kalehe clustered together in the haplogroup A (**Figure 5.3**).
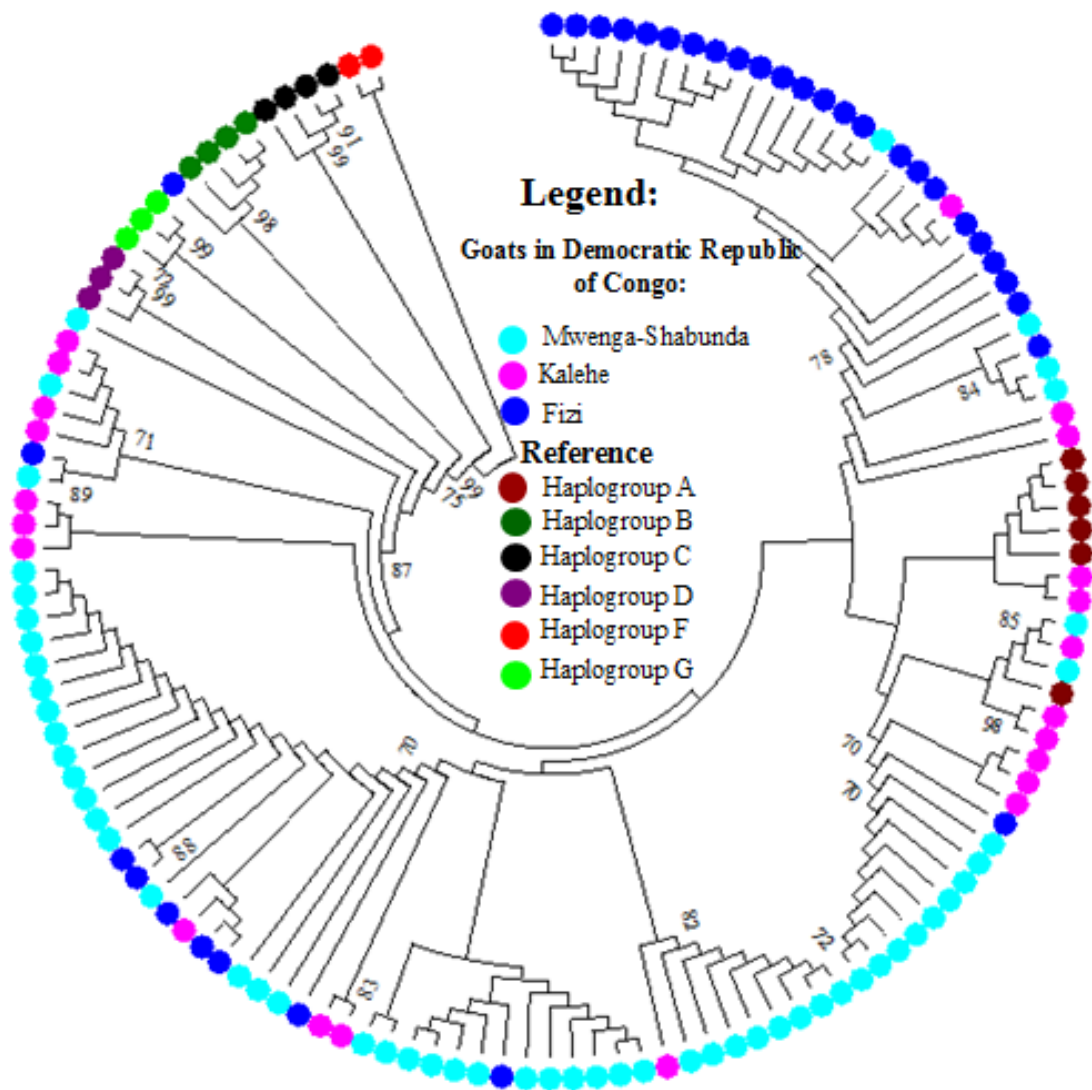
Figure 5.2: Neighbour joining (NJ) tree of the three Democratic Republic of Congo goat populations, six reference haplogroups.
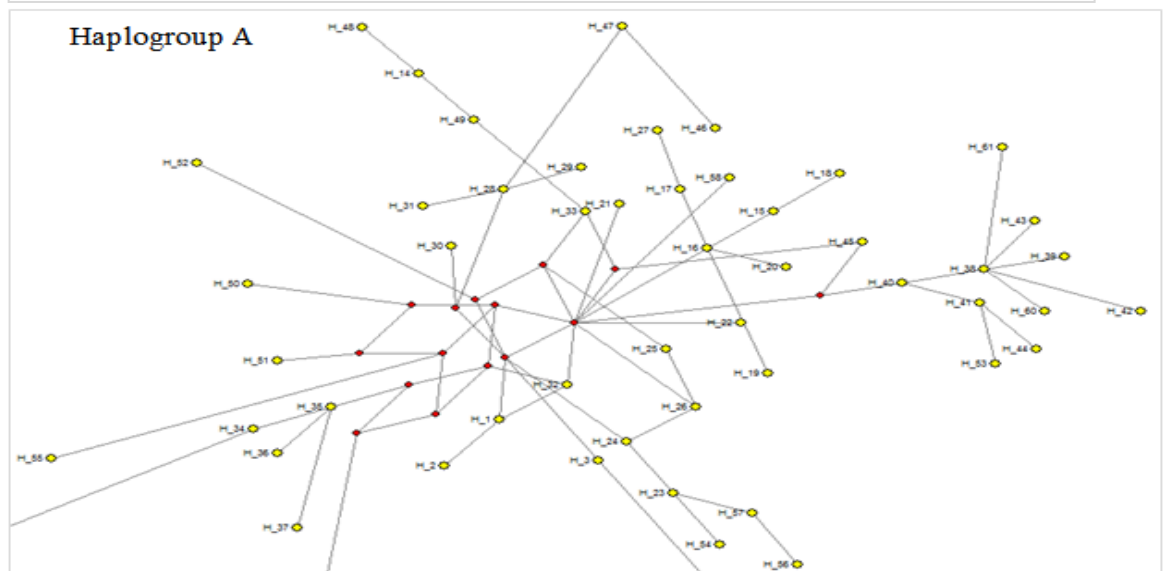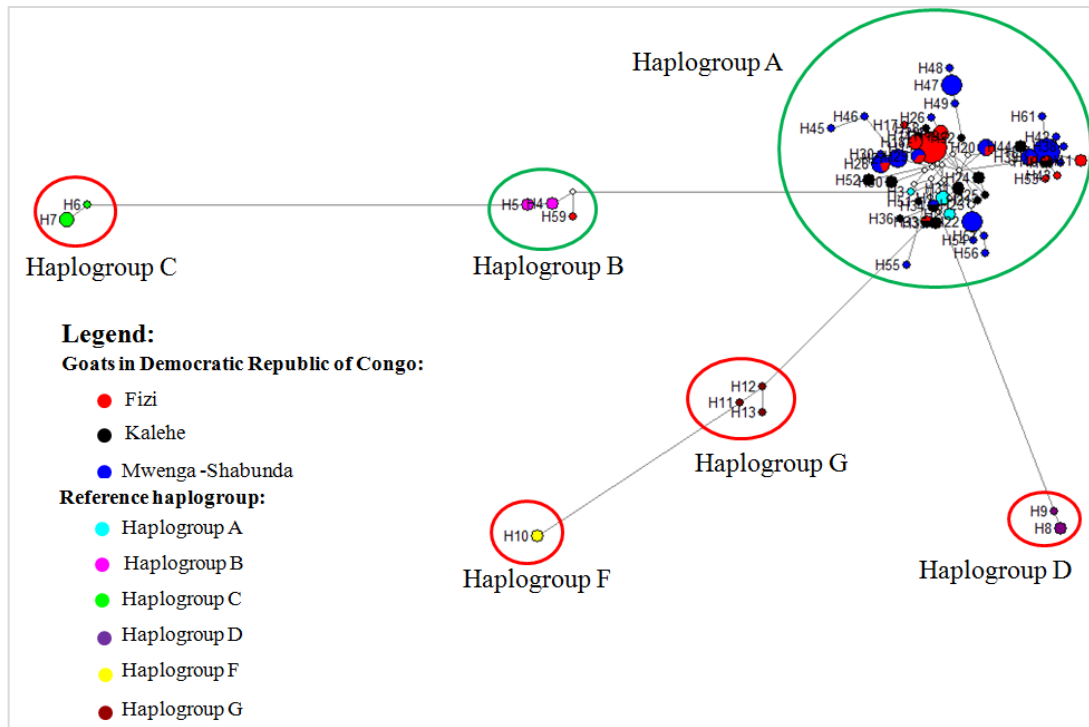
Figure 5.3: Median-joining (MJ) network for the 56 goats mtDNA haplotypes by red and yellow circles.

5.3.4 Population differentiation

The results from analysis of molecular variance (AMOVA), shown that 83.22% of goat's genetic variation present in indigenous goats from a PPR outbreak in South Kivu, Democratic Republic of Congo, was explicated by individuals genetic dissimilarities within populations, 11.18% of total variation (P=0.0000) was explained by the differences among groups and only 5.6% (P=0.00286) was explained by the variation among population within groups (**Table 5.2**). The pair-wise comparison values (F_{ST}) of 0.05273 were found between Kalehe and Shabunda-Mwenga indigenous goats, 0.18573 between Fizi and Shabunda-Mwenga and 0.16004 between Fizi and Kalehe (**Table 5.3**).

Table 5.2: Molecular Variance based on haplogroups and population groupings.

Source of variation	d.f	Sum of squares (SSD)	Variance components	Percentage of variation	*p-value*
Among groups	1	50.875	0.71999 Va	11.18	0.0000*
Among populations within groups	1	16.682	0.36031 Vb	5.60	0.00286*
Within populations	108	567.920	5.35774 Vc	83.22	0.32658[ns]
Total	**110**	**635.477**	**6.43804**	-	-

Key: *= significant at p<0.05; SSD=Sum of square deviation, V=Variance components and ns=non-significant.

Table 5.3: Pairwise distance matrix (F_{ST}) of the goat populations studied

Population	Shabunda_Mwenga	Kalehe	Fizi
Shabunda_Mwenga	0.00000	-	-
Kalehe	0.05273	0.00000	-
Fizi	0.18573	0.16004	0.00000

5.3.5 Population dynamics

To assess population expansion events and mismatch distributions, Fu's Fs statistic were used in this study and the results showed the bimodal mismatch distribution in each population characterized (**Figure 5.4**). The observed pattern for Shabunda-Mwenga and Kalehe goat population, did not differ significantly from one expected for expansion, but it differs from the Fizi goat's population of Fizi (**Table 5.3**). Tajima's D and Fu's F_S statistics tests (**Table 5.4**) showing presence of the population expansion supported these results.

Table 5.4: Population demographic and neutrality test in the Democratic Republic of Congo goat populations

Population	N	S	SDD	Raggedness index "*r*"	Tajima's D (p)	Fu's FS (p)
Shabunda_Mwenga	55	71	0.01841024	0.01296376	0.24000	0.33300
Kalehe	22	65	0.00896673	0.01413017	0.03900	0.20900
Fizi	32	42	0.01283777	0.03094108	0.06600	0.32200

Key: SSD=Sum of square deviation; N=Number of sequences; S=Segregating site;*= significant at p<0.0; **=significant at p<0.01; ns=non-significant
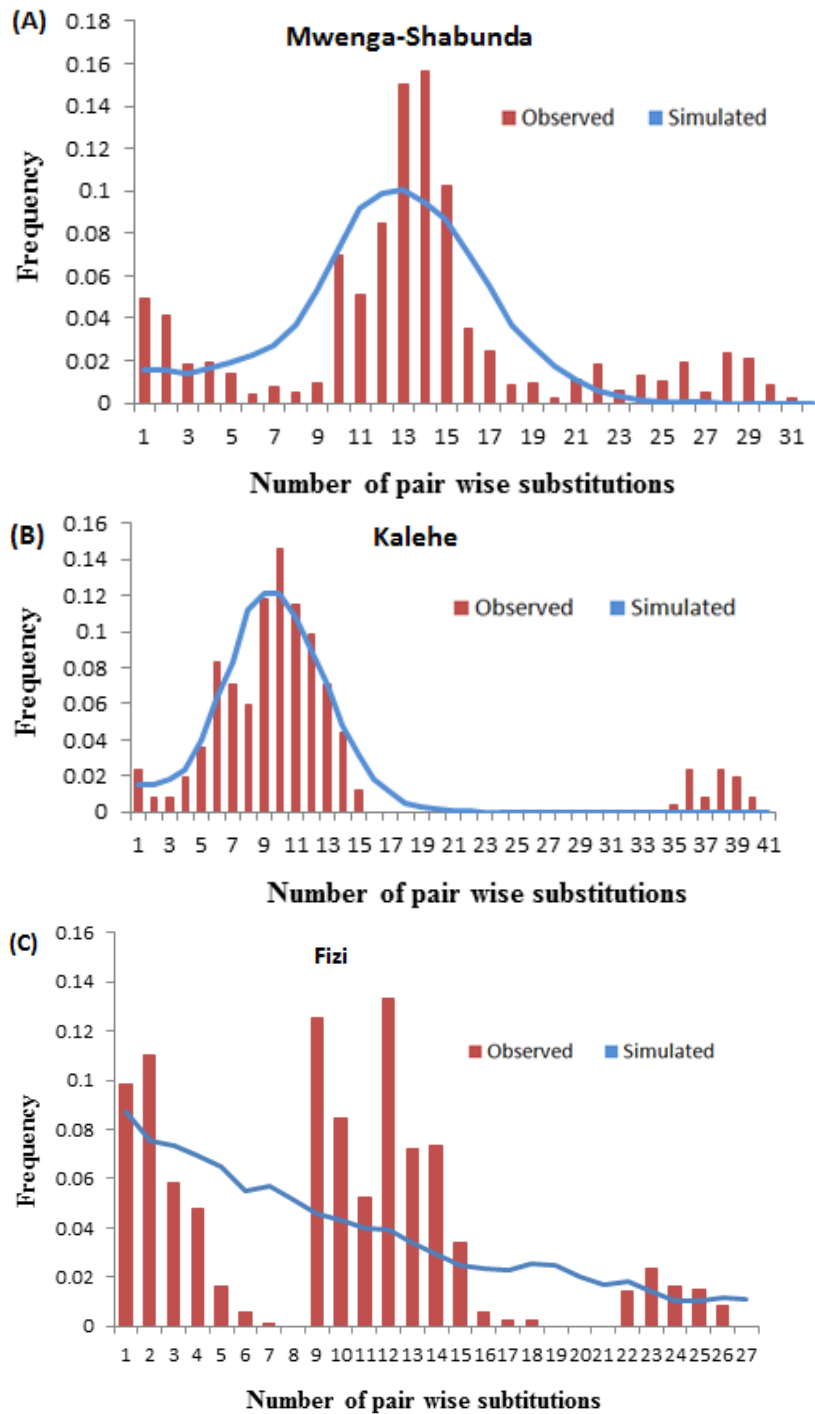
Figure 5.4: Mitochondrial DNA haplogroups mismatch distributions for of South Kivu indigenous goats.

5.4. DISCUSSION

In this study, the investigation of genetic diversity in the control region of mtDNA (*d-loop*) and assessment of the DRCongo goats population origin. High genetic diversity were detected from 56 haplotypes in a 1,220-bp sequence of 111 sequenced animals and three goat populations studied that included Shabunda-Mwenga, Kalehe and Fizi and two maternal haplogroups (A and B). Naderi *et al*. (2007) reported haplotype B in few populations of Namibia and South African goats. Several authors in previous studies found multiple maternal haplogroups of domestic goat (Luikart *et al.,* 2001; Sultana *et al.,* 2003; Chen *et al.,* 2005; Naderi *et al.,* 2007). These findings of mtDNA haplotypes cluster in six haplogroups including A-B-C-D-F and G are supported with the MJ network analysis that was obtained from this study (**Figure 5.3**), with the predominance of haplogroup A. The few number of median vectors observed in this study compared to literature gives clues that there could be animals which were not sampled or were extinct among Fizi goat's population. Naderi *et al.* (2007) showed that haplogroup B was more dominant in Southern African countries, while haplogroup A was widely distributed with high frequencies found in North and Latin America. Haplogroup C was reported only in Italy /Siri and Tukey recently, while haplogroup D was reported mostly in Eastern Asia, including Southern part, India, Pakistan and China. Finally, haplogroup G was mostly found in Eastern Africa, Egypt, South Arabia, Turkey, Iraq, Iran, India and Pakistan.

It was found that, globally, there was a low phylogeographic structure among domestic goats (Naderi *et al.*, 2007). Haplotypes which were present in D R Congo in the past

(Gene pool of original *Caprine*) but were not samples or extended after their introduction into the country are presented by the median vectors found.

Higher rate/level of genetic diversity was observed in Haplogroup A compared to haplogroup B. A total of 10.7 nucleotide differences, 120 segregating sites and 124 mutations. Similar results were reported in three goat populations in Morocco by Benjelloun *et al.* (2015), whereby 64 polymorphic sites were detected and 40 haplotypes belonged to haplogroup A. Previous, Colli *et al.* (2015) found in goat matrilineal variability 229 polymorphic sites in the whole mitochondrial genome analyses.

Moreover, 83.22% of the studied goat's total genetic variability was due to genetic dissimilarities between individuals in the populations, 11.18% among groups and only 5.60% of the variabilities could be attributable to genetic dissimilarities between populations (**Table 5.2**). This estimation is similar to previous findings where 83% of the variation within populations were observed in Indian goats, but differed from SCA goats where 69% of variation was observed within populations and 78.7% for goats originated from Africa, Europe and Asia (Amills *et al.,* 2004; Amills *et al.,* 2008). At the global level (54 countries included), Naderi *et al.* (2007) observed 77% of genetic variation in goats through mitochondrial analysis. The variations among breeds within and among geographic locations showing a weak phylogenetic structure due to the global coverage of haplogroup A was explained at 11 and 5.5.6 % respectively.

The haplotype diversity of 0.971 obtained in the current study (**Table 5.1**) is low compared to the estimate of haplotype diversity for Iberian goats (0.996) and European goats (0.994).

However, in this study, the haplotype diversity in goat's population was higher than estimates of South and Central American goat's haplotype diversity (0.963), Sicily (0.806-0.969) and for Atlantic goat populations (0.965) as reported by Amills *et al.* (2004 and 2008). About 0.9884 of haplotype diversity estimates have been reported for goat lineage mtDNA sequence analyses (Naderi *et al.,* 2007). Subsequently in the same year Naderi *et al.* (2007) observed a high rate of variable sites in a 558 bp of HVI region alignment sequences of about 336 variable regions. Simultaneously, lowest estimates nucleotide diversity of 0.873±0.051 was detected in Kalehe population compared to the rest of D.R. Congo goats. This statement was confirmed with the phylogenetic network, F_{ST} and populations admixture analyses presented in **Table 5.3**, **Figures 5.3** and **5.4.**

This could be explicated in one hand by a minimal gene flow level towards Kalehe region and on the other hand Kalehe goat habitat must be unfavorable for other goats from D R Congo to adapt to the local environment conditions.

The analysis of population expansion showing by the bimodal distributions observed in the graph in the current study indicated the occurrence at some time in the history of one major and one minor population expansion events in D.R. Congo (**Figure 5.4**). The positive and non-significant *F*s values obtained in this study confirmed a slow population expansion (**Table 5.4**). Previously, Chen *et al.* (2005) observed the negative

Fs estimate (Fs = -23.57; p<0.01) for Chinese goats. Human cultural and socio-economic connections might explain the demographic expansion of D R Congo goat populations. Recently, the curves of multimodal mismatch were found in Anatolian Black and Angora populations (Akis *et al.,* 2014).

In conclusion, this study has revealed a very high mtDNA control region fragment differentiation between Shabunda-Mwenga, Kalehe and Fizi goat breeds which had previously suffered from a *Peste des petits ruminants'* outbreaks. The results also proved that the three populations have undergone population expansion in the past, which reflects differences in their demographic histories. Majority of the studied goats in South Kivu were clustered into haplogroup A and only few sequences in haplogroup B. This might be probably due to the human population movement or migration for various reasons and especially in Fizi goat populations.

CHAPTER SIX: MIXED INFECTION OF PESTE DES PETITS RUMINANTS AND CAPRIPOX IN GOATS AND SHEEP IN SOUTH KIVU: INTERFERENCE WITH RESPECTIVE DIAGNOSES

6.1 INTRODUCTION

Respiratory diseases show common clinical symptoms in small ruminants, like pneumonia, and are caused by different types of pathogens: viruses, bacteria and parasites in single or multiple infections (Ozmen *et al.,* 2009; Chu *et al.,* 2011; Saeed *et al.,* 2015). An appropriate identification of the actual pathogen(s) responsible for the disease is critical for timely and proper management of those diseases. Previous studies from Thiaucourt *et al.* (1996), Harper *et al.* (2006), Odugbo *et al.* (2006) and Valadan *et al.* (2014) showed that the major pathogens responsible for respiratory syndromes of sheep and goats in Africa, Middle-Est and Asia, include Capripoxvirus (CaPV), *Peste-des-petits ruminants* virus (PPRV), *Pasteurella multocida* (PM), *Mycoplasma capricolum* subspecies (ssp.) and *capripneumoniae* (Mccp). Detection of these diseases is routinely done using Enzyme linked Immunosorbent Assays (ELISA) or microbiological methods which are currently being replaced by molecular based detection methods including multiplex assays, where detection of different pathogens is carried out in a single reaction (Settypalli *et al.,* 2016). However, although multiplex tests for detection of different pathogens in a single reaction aid in a more rapid diagnosis, they tend to present a few disadvantages such as complexity in handling the protocol and the requirement of advanced technologies such as microarrays, liquid arrays and mass spectrophotometers. Babiuk *et al.* (2008) and WOAH (2015) also

confirmed the symptoms including fever, skin and mucous membrane lesions, conjunctivitis and sometimes breathing distress in both PPR and CaP diseases. Previous reports from Banyard *et al.* (2010), Brown (2011) and Libeau *et al.* (2014) confirmed that the two endemic diseases co-infect. A third complication may be brought in by Lumpy skin disease virus (LSDV), which infects mostly cattle (Santhamani *et al.,* 2014). However, Tuppurainen *et al.* (2017) has shown that some viral strains may replicate in goat and sheep. Several investigators like Jiang *et al*. (2010), Gao *et al.* (2011), Thonur et *al.* (2012) and Venkatesan et *al.* (2014) have previously developed PCR and RT-PCR based multiplex protocols for the differential detection of closely related pathogens or pathogens causing diseases with similar symptoms. Lamien *et al.* (20110) and Gelaye *et al.* (2013) were able to detect 250 copies and 20 copies of CaPV by using the classical PCR and real time PCR methods, respectively. In this study, conventional PCR and reverse transcriptase PCR detection methods were used for CaPV and PPRV, respectively, in different tubes. The objective of this study was to describe a mixed infection of PPRV and CaPV causing respiratory infections in small ruminants in South Kivu in a single tube, one and two-step reaction.

6.2 MATERIALS AND METHODS

6.2.1 Study area

This remained as given in Section 3.2.1 above.

6.2.2 Sampling and sample processing

There was purposive selection of 150 small ruminants (goats and sheep) which presented both capripox and *Peste-des-petits ruminants* common clinical signs including fever, skin and mucous membrane pox lesions, conjunctivitis and sometimes breathing distress, self-resolving, muzzle and lips lesions, serious mucopurulent nasal and ocular discharge, diarrhea, lacrimation, matting of eye lids, cutaneous nodules, erosions on the soft palate and gums, labored breathing. Samples were obtained and processed as previously described in chapter 4, section **4.2.2** and **4.2.3.**

6.2.3 Sample analysis

6.2.3.1 DNA extraction

Viral DNA extraction was carried out from the 150 goat and sheep samples as previously used to detect PPRV and described in **chapter 4**. The DNA was extracted from ground and homogenized tissue and swab samples using the DNeasy extraction kit (Qiagen, Hilden, Germany, Model: 69506) following manufacturer's procedure (**Appendix 14**). Similarly, viral DNA was extracted from frozen blood samples using salt extraction method where 1 ml of blood was mixed with 4 mls of lysis buffer (Sucrose:109.536g, 1M Tris-HCL(P^H 7.5):10mls, 1M MgCl2: 5ml and Triton-X 100 (Add last): 10mls to suspend the white pellet (**Appendix 15**). The quality and quantity checking of the viral DNA was as was done for extracted viral RNA (described in chapter 4, Section 4.2.4.2).

6.2.3.2 Detection of CaPV by PCR

To amplify the GPCR, RPO30 and P32 genes of CaPV genome, the viral DNA extracted was used was used. The *P32* gene corresponds to a capsid protein encoded by Vaccinia virus H3L gene and it is located on the mature intracellular viral particle surface membrane (Tulman *et al*., 2002). The GPCR gene encodes for receptors of G protein-coupled chemokine, and his structure has in the first and second extracellular loops seven hydrophobic sites and the cysteine residues (Cao *et al*., 1995). The RNA polymerase subunit gene *(RPO30)* encodes the 30 kilodalton DNA-dependant RNA polymerase and it is a homologue of the Vaccinia virus E4L gene (Lamien *et al*., 2011). These three genes are highly conserved among capripox viruses and that is the reason why their sequences are useful in SPP, GTP and LSD viruses' differentiation (Zhou *et al*., 2012).

The first set of primers that was used included: *P32*-1 (5'…ATG GCA GAT ATC CCA TT…3') and *P32*-2 (5'…TTA CCA CAG GCT ATT AGA AG…3') which amplified up to 990 bp fragments enclosing the complete *P32* ORF (Open Reading Frame). The second set of primers included: *GPCR*-1 (5'…TTT ATC AGC ACT AGG TCA TTA TCT…3') and *GPCR*-2 (5'…TAT CAC TCC CTT CCA TTT TTA T…3') which amplified 1100 bp fragment comprising the complete *GPCR* ORF. The third set of primers included: *RPO30*-1 (5'…CTC TGT TCC AAA CTA AAT CAT…3') and *RPO30*-2 (5'…TTT TTG TAT TAC CAA TTT CTG…3') which amplified 987 bp fragment covering the complete *RPO30* ORF as suggested by Zhou *et al.* (2012).

For the reaction of PCR, a total volume of 100 μl (20 ng of DNA extracted, 20 μl 5 ×

buffer PS, 1 μl of PrimeSTAR® DNA polymerase, 8 μl 2.5 mM of dNTP, 63 μl of water

nuclease-free, 0.20 μM of each primer. The amplification of DNA was executed by the

same thermal Cycler (GeneAmp PCR System 9700). Cycling conditions were: initial

denaturation at 98° C for 10 seconds, 35 cycles of: 10 seconds denaturation at 98° C 20

seconds of annealing at 47° C and 120 seconds of extension at 72° C. A final extension

was followed for 20 minutes at 72° C.

6.2.3.3 Gel electrophoresis and visualization

This was as given in Chapter 4, Section 4.2.4.4

6.2.3.4 PCR products purification

The PCR products were purified using QIAGEN kit (QIAquick® Gel Extraction Kit:

cat. nos. 28704 and 28706) following manufacturer's instructions (**Appendix 9 and 10**).

Briefly, the amplified DNA section was excised from the agarose gel with a sterile

sharp-scalpel, weighted in a colorless tube, before adding 3 volumes QG Buffer to 1

volume of the gel, then passed through QIAquick spin column before washing with

appropriate PE buffers and eluding with water free nuclease of buffer EB provided in

the kit in a clean collection tube.

6.2.3.5 Sequencing

Capripox virus positive samples for all the three genes (*P32, RPO30* and *GPCR*) were

sequenced directly using the kit of Big Dye Terminator Cycle Sequencing v3.1

following the manufacturer's instructions (**Appendix 11**). The PPRV RNA sequencing was as given in Chapter 4, Section 4.2.4.5.

6.2.4 Data analysis

Microsoft Office Excel 2016 was used to calculate frequencies of PPRV found in chapter 4 and Capripox samples prevalence by using a formula of proportion:

$$p = \text{\#positive samples/Total sample};$$

$$\text{Where p is prevalence}$$

Homologous *P32, GPCR* and *RPO30* CaPV and the F-PPRV nucleotide sequences genes were gotten from the GenBank with the online Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) to identify regions of local similarity between sequences. The Molecular Evolutionary Genetics Analysis (MEGA 6), version 6 helped to perform multiple-alignment of these sequences with ClustalW method as suggested by Nei *et al.* (2000) using Neighbor-Joining (NJ) and Maximum Likelihood (MJ) tests and Hasegawa-Kishino-Yano (HKY) model for *RPO30* gene, Tamura 3-parameter (T92) model for fusion PPRV gene, *GPCR* and *P32* genes to perform phylogenetic analysis. In order to get good reliable phylogenetic trees the bootstrap analysis with 1000 replicates were runned (Tamura *et al.,* 2013).

6.3 RESULTS

6.3.1 Qualification and quantification of extracted DNA

Similar to the quality of RNA found in chapter four, many of the tested goat and sheep samples had a very good DNA quality, represented by a tight-band without or with minimal smears and having > 23kb of molecular weight and closer to the both DNA lambda (**λ**) and DNA positive control sample (PC) (**Figure 6.1**).
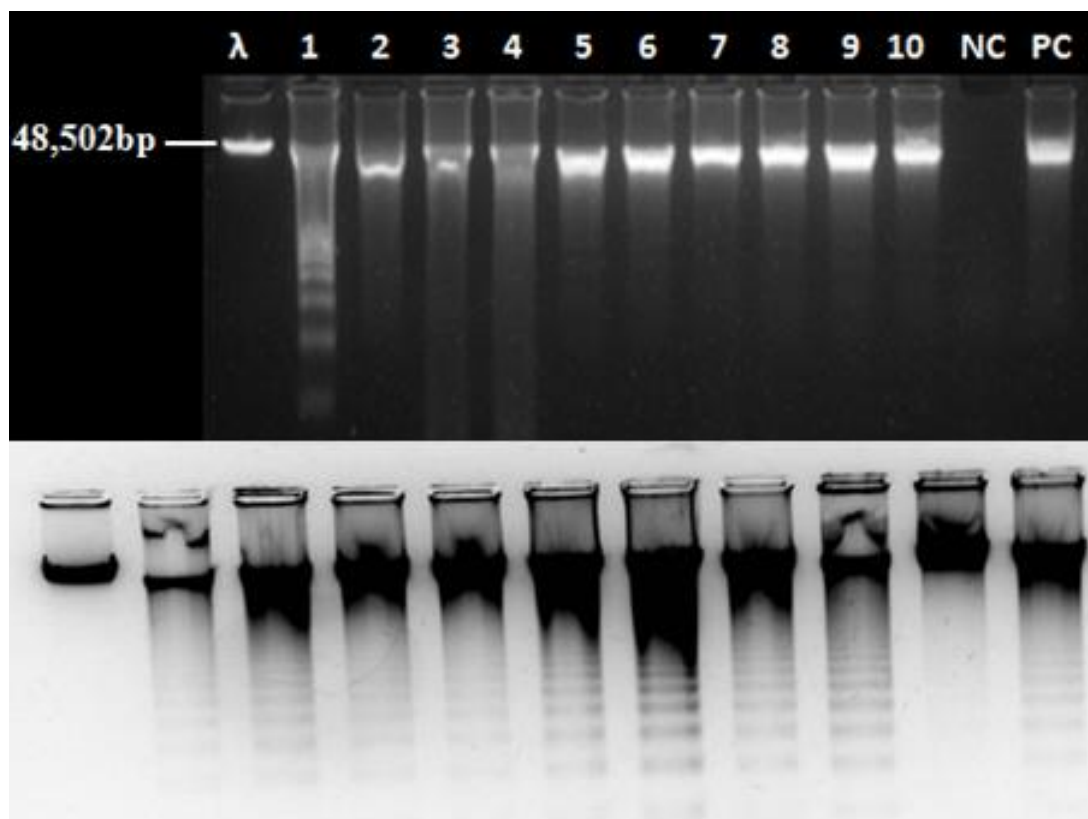
Figure 6.1: Electrophoresis gel image of genomic DNA visualized under transilluminator UV. NC represents the negative control, PC the positive control, 1-10 the DNA samples and **λ** (the lambda genomic DNA representing the DNA marker).

The genomic DNA concentrations were very high compared with the total RNA concentrations presented in chapter 4, section 4.3.1 (**Table 4.3** and **appendix 13**) and

varied from 7.5 (sample 9) to 1892.38 ng/µl (sample 108), with an average of >100ng/µl when all the extracted samples were considered, which is good for diagnostic purposes (**Table 6.1** and **Appendix 16**). Similarly, the nucleic acid purity ratio (260/280 and 260/230) for many of the samples used approached 2, which ensured the purity of the extracted total DNA (**Table 6.1**and **Figure 6.2 A, B, C and D**).

Table 6.1: Genomic DNA concentrations and purity with NanoDrop® ND-100.

Sample ID	NA (ng/µl)	A260	A280	260/280	260/230	Factor
Minimum	7.5	0.151	0.06	2.52	0.56	50
Maximum	1892.38	37.848	20.744	1.82	1.66	50

NA: Nucleic Acid concentration

(A)

(B)

Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor

Wave length (nm)

131

(C)

Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor

Wave length (nm)

132
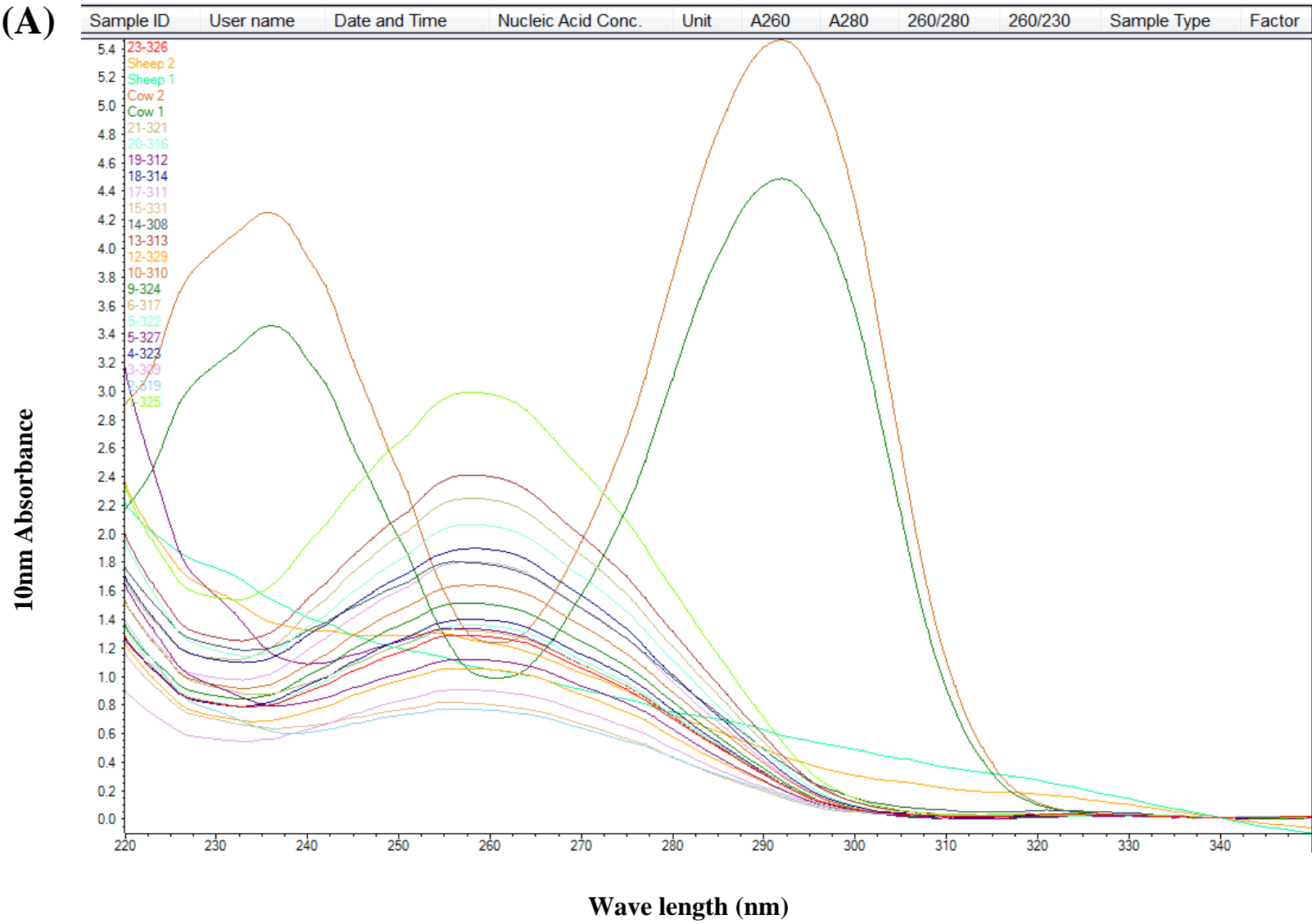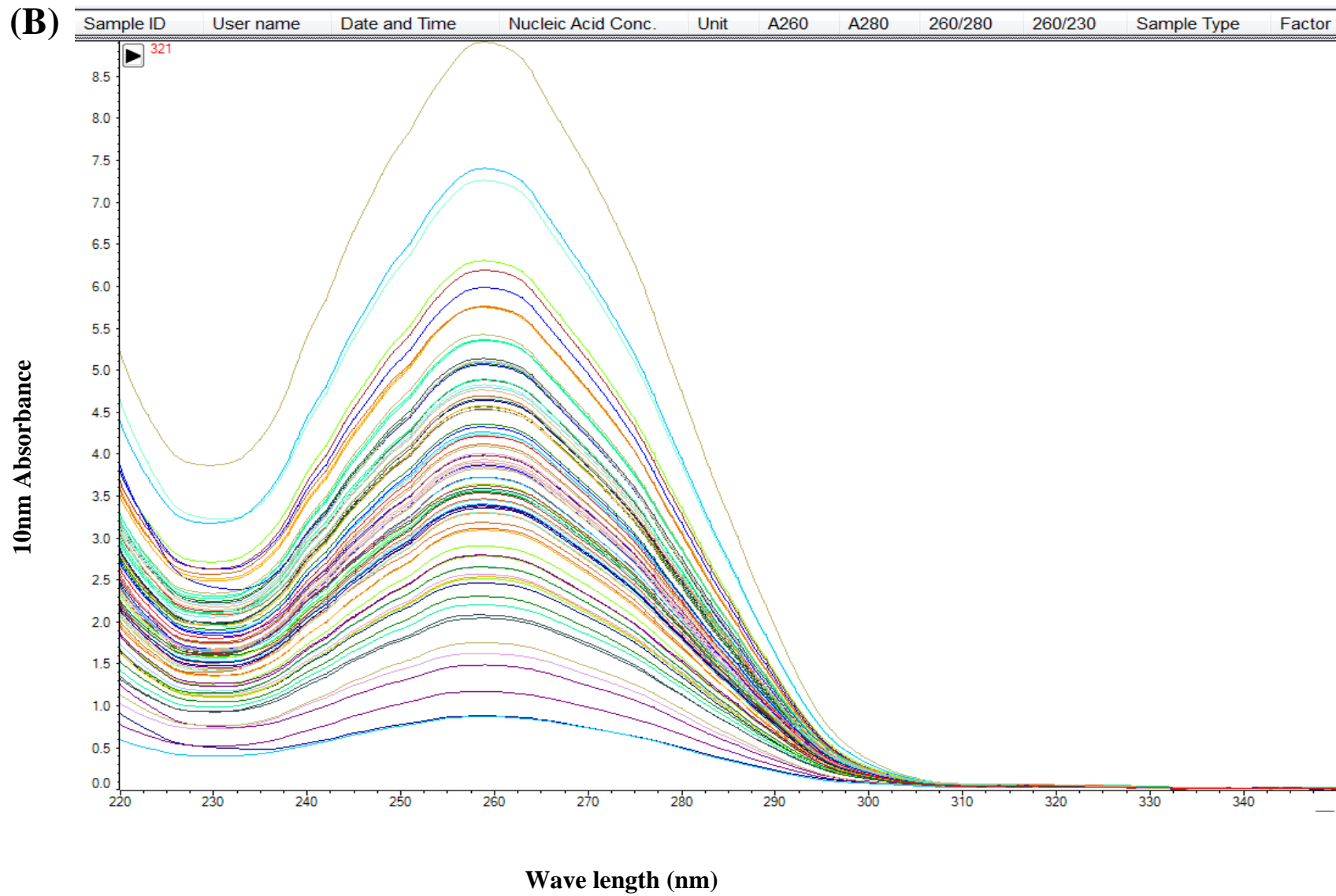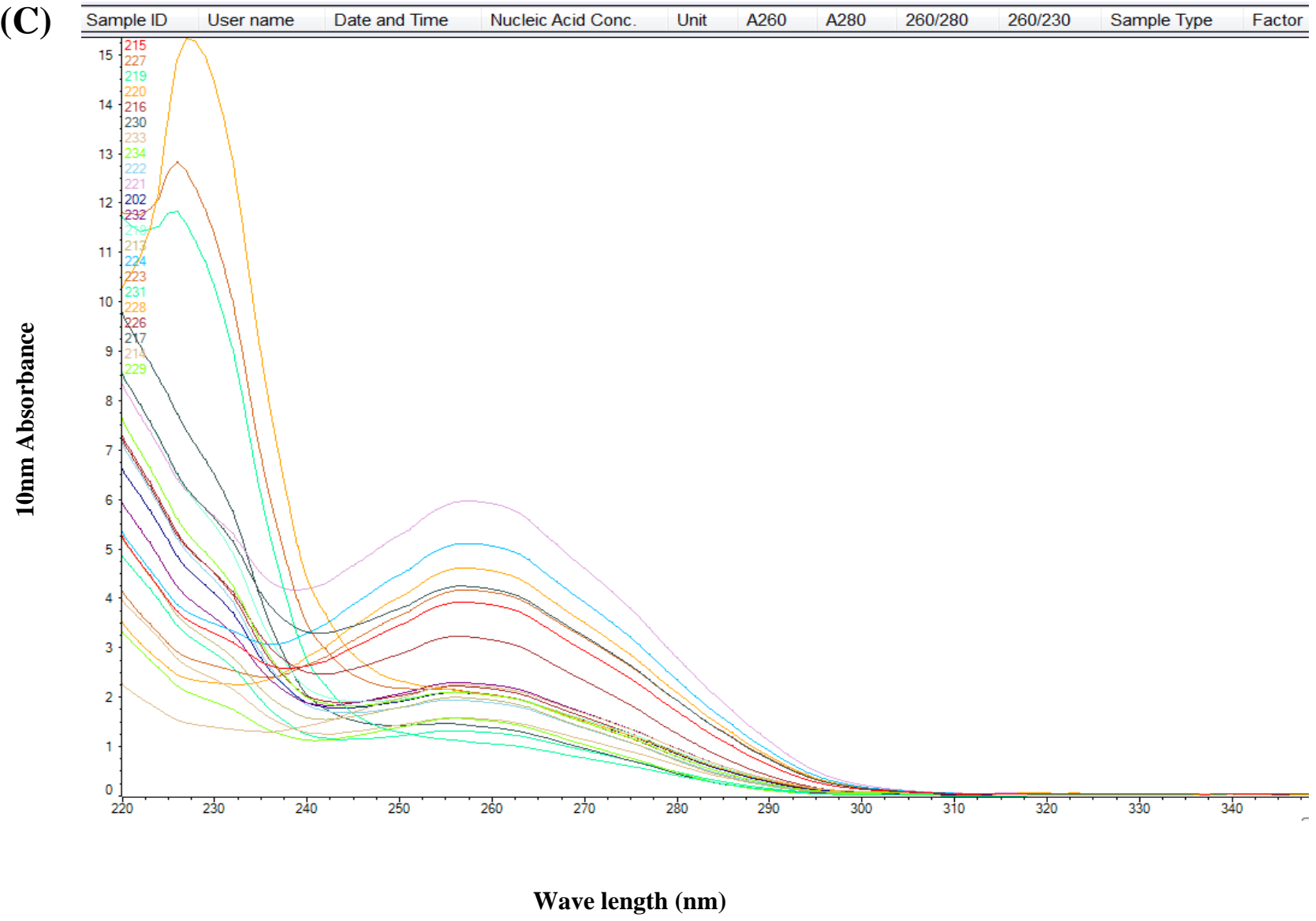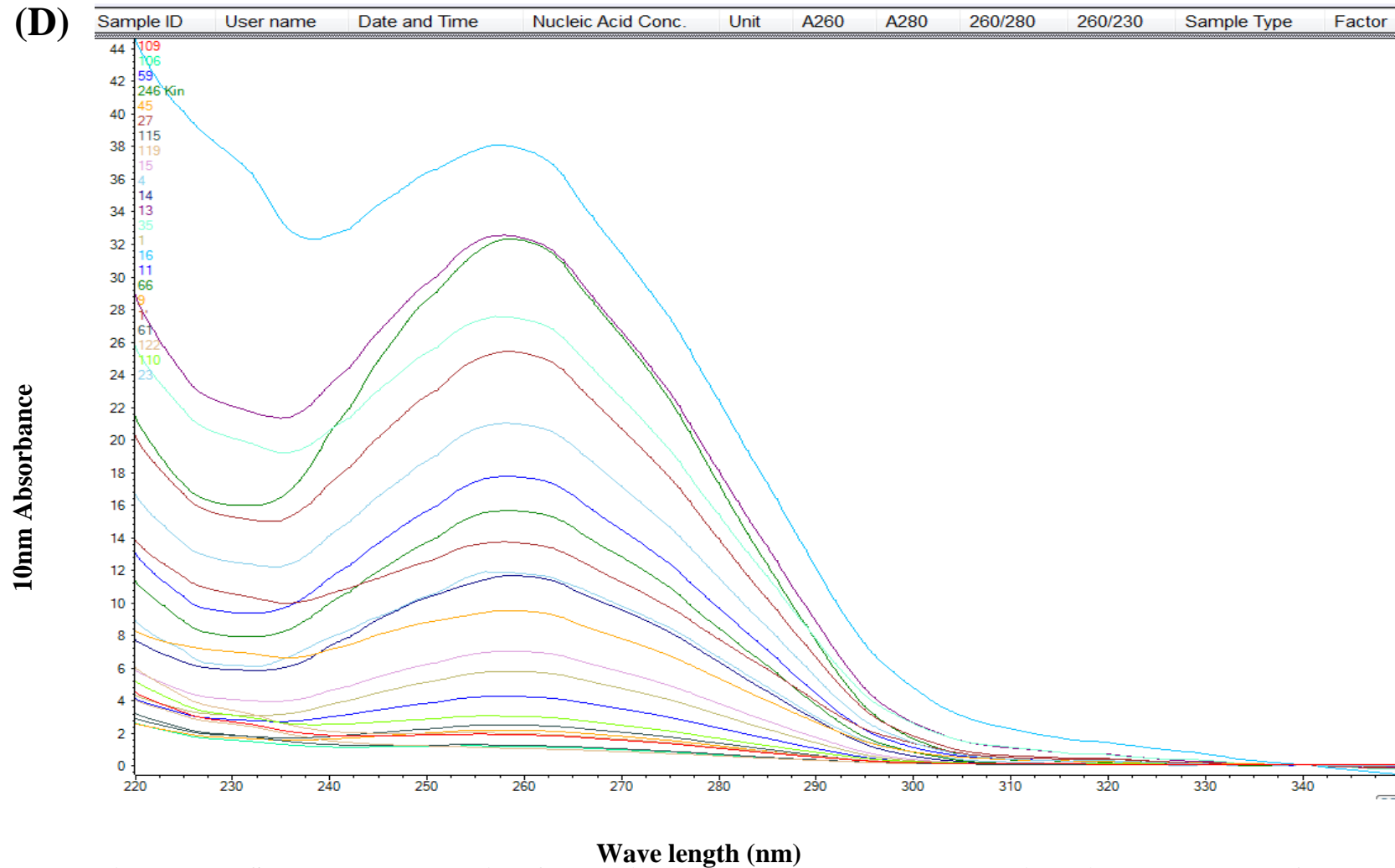
Figure 6.2: Spectral presentation for DNA samples absorbance and purity with a NanoDrop® ND-1000(A, B, C and D).

6.3.2 Detection of capripox viruses with PCR

6.3.2.1 *P32* Gene

Amplicon of 990 bp of the *P32* capripox virus gene which possesses the specific signatures for both GTP and SPP viruses at nucleotide and amino acid sequences level were amplified and are shown in tight white bands (**Figure 6.3**).
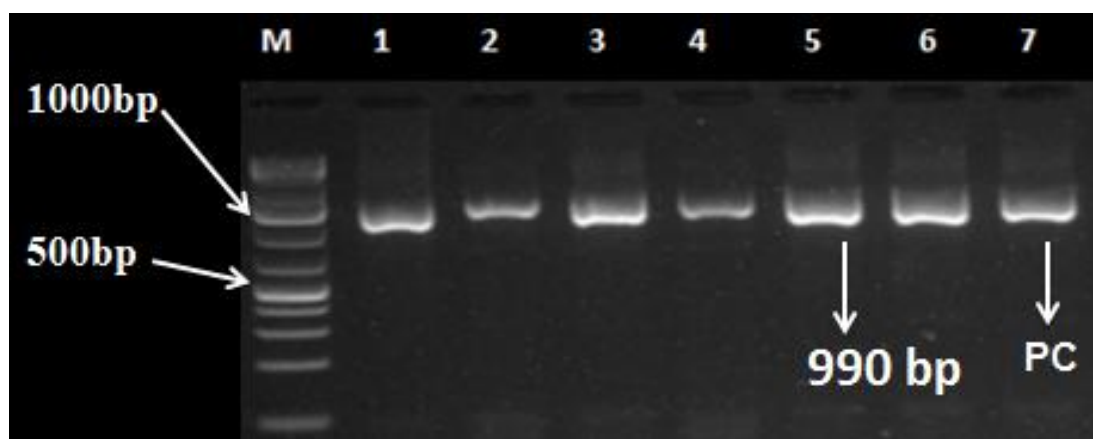
Figure 6.3: PCR products visualized under UV transilluminator showing *P32* gene amplicons of Capripox virus. M is the DNA marker (1kb plus), 1-6 samples, 7 is a positive control.

6.3.2.2 *RPO30* Gene

Similarly, a fragment of 987 base pairs of *RPO30* gene of capripox virus which plays almost the same role as the previously described *P32* gene was amplified. Positive samples are presented as tight white bands (**Figure 6.4**).
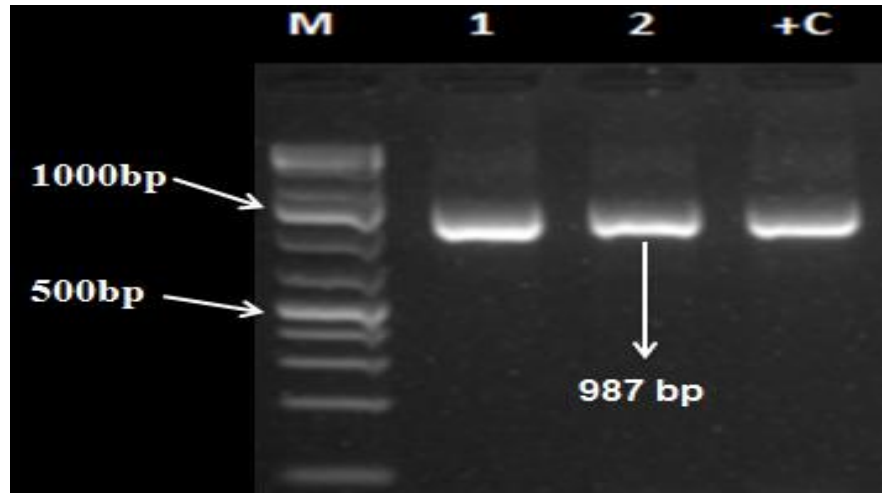
Figure 6.4: PCR products of *RPO30*gene amplicons of Capripox virus.

6.3.2.3 *GPCR* Gene

The amplicon of 1100 bp was amplified from the GPCR gene of capripox virus. Positive samples are shown as tight white bands (**Figure 6.5**).
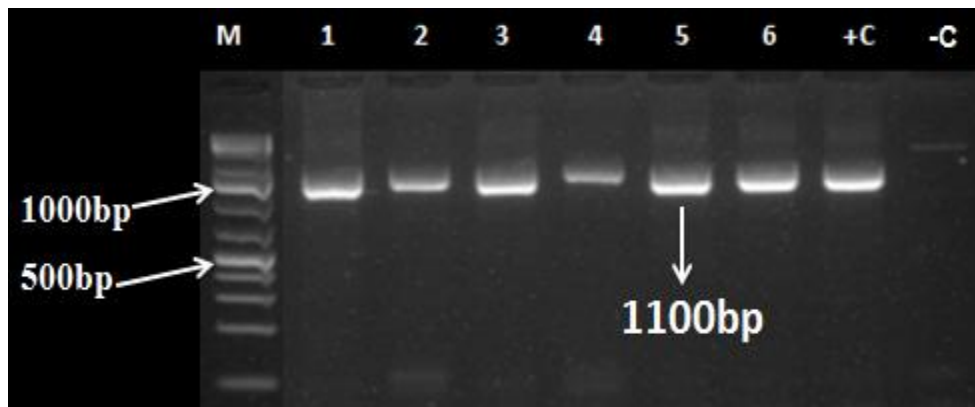
Figure 6.5: PCR products of *GPCR* gene amplicons of Capripox virus.

6.3.3 Mixed infection prevalence of CaPV and PPRV

Among a total of 150 samples (120 from goats and 30 from sheep), an overall PPRV prevalence of 64.7% (97/150) was observed and 52.7% (79/150) prevalence of capripox was recovered (**Table 6.2**). A total of 58 animals out of the 150 tested (38.7%) were infected with both PPRV and CaPV (**Table 6.2**). The high mixed prevalence of PPRV and CaPV was found in Mwenga and Shabunda regions at 54.3% (19/35) and 53.5% (21/40), respectively, followed by Fizi region at 45% (18/40). No CaPV-PPRV coinfection was found in small ruminant samples from Kalehe region despite the high capripox prevalence (37.1%) observed (**Table 6.2**).

Table 6.2:Co-infection prevalence between CaPV and PPRV

Location	Samples tested	PPRV +Ve	PPRV (%)	CaPV +Ve	CaPV +Ve (%)	PPRV-CaPV +Ve	PPRV-CaPV +Ve (%)
Shabunda	40	35	87.5	24	60	21	52.5
Mwenga	35	27	77.1	20	57	19	54.3
Fizi	40	28	70	22	55	18	45
Kalehe	35	0	0	13	37.1	0	0
Total	**150**	**97**	**64.7**	**79**	**52.7**	**58**	**38.7**

Legend: CaPV=Capripox virus, PPRV=*Peste des petits ruminants virus*, +Ve=Positive.

6.3.4 Phylogenetic analysis of capripox virus

The *RPO30*, *GPCR* and *P32* gene sequence analyses were used for differentiation of SPPV, GTPV and LSDV. All the samples from South Kivu province of D R Congo

were clustered under LSDV lineage as presented in the three phylogenetic reconstructions shown in **Figure 6.6**; **Figure 6.7** and **Figure 6.8**.

6.3.4.1 Phylogeny of *P32* gene of capripox virus

The capripox virus detected and characterized in goats and sheep from South Kivu province of D. R. Congo, particularly in Shabunda and Mwenga based on the sequencing of partial *P32* gene sequencing clustered within LSDV Lineage which differed genetically with the FTPV and SPPV (**Figure 6.6**). All the phylogenetic trees were inferred following 1000 bootstrap replications.
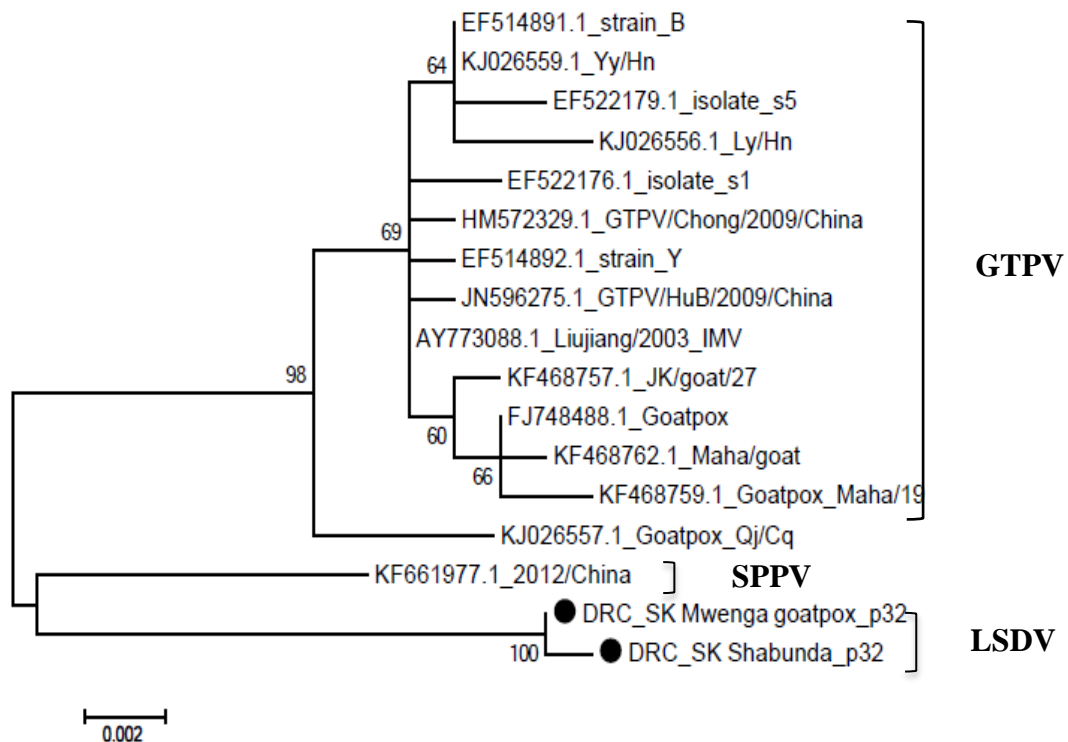
Figure 6.6: Phylogenetic relationship of *P32* gene of Capripox virus.

A Maximum-Likelihood phylogenetic tree depicting the relationship of D. R. Congo CaPV obtained from this study (indicated with black dot) with other CaPV belonging to the three CaPV Lineages (GTPV, SPPV and LSDV). Phylogeny was inferred following 1000 bootstrap replications and values <50% were not shown.

6.3.4.2 Phylogeny of *RPO30* gene of capripox virus

The south Kivu capripox sequences of the *RPO30* gene was almost similar at 99% with that of lumpy skin disease virus RSA/54 Haden RNA polymerase isolate in South Africa with the accession number of GU119937.1 in NCBI gene Bank, which are clustered into the lineage of lumpy skin disease virus. South Kivu province isolates, including those from Shabunda and Mwenga, were not 100% identical and were clustered within LSDV Lineage (**Figure 6.7**).
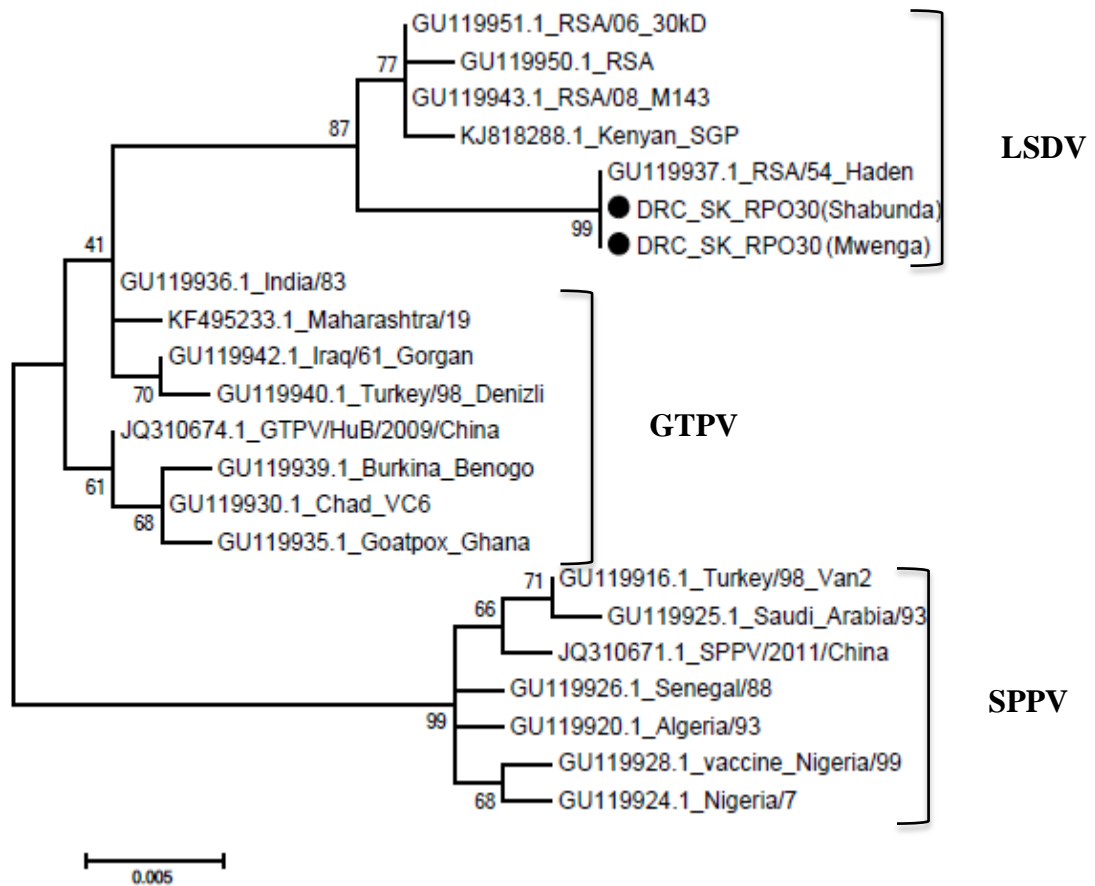
Figure 6.7: Phylogenetic relationship of RPO30 gene of Capripox virus.
A Maximum-Likelihood phylogenetic tree depicting the relationship of D R Congo CaPV obtained from this study indicated with black dot together with other CaPV lineages.

6.3.4.3 Phylogeny of *GPCR* gene of capripox virus

Capripox virus found in South Kivu province (Shabunda and Mwenga) are not 100% identical based on the partial sequencing of 1100 amplicon and are clustered within LSDV Lineage, the sub group II closer to RSA capripox isolates (FJ869376.1) with rate of evolutionary change 92% as shown by branch lengths in the trees (**Figure 6.8**).
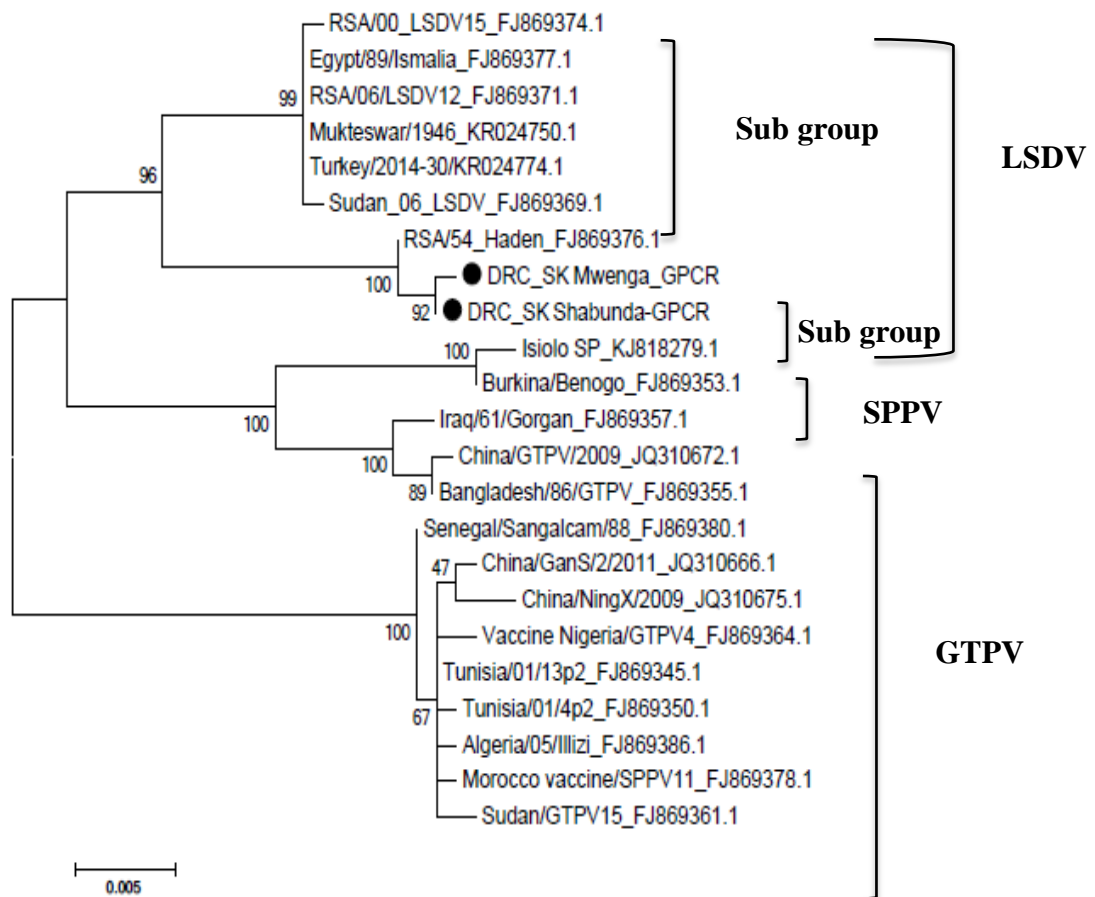
Figure 6.8: Phylogenetic tree of GPCR gene of Capripox virus. A Maximum-Likelihood phylogenetic tree depicting the relationship of the D R Congo CaPV obtained from this study (indicated with black dot) with other CaPV Lineages.

6.3.5 Genetic and Phylogenetic relationship of CaPV-*P32*gene and PPRV-F gene

Four sequences from goat samples in South Kivu, out of which two were from the fusion protein of PPRV and the other two from *P32* gene of CaPV, were aligned for phylogenetic analysis and each sequence was compared to each other for genetic similarity. The PPRV Fusion gene obtained in Mwenga region was 47.17% identical to the CaPV-P32 gene in trimmed sequences fragment of 720 bp from the same location

during a PPRV-CaPV co-infection in goats after alignments. The PPPV fusion gene sequence obtained in Shabunda was 48.65% identical compared to the *P32* gene of CaPV found in a CaPV-PPRV mixed infection in goats in that same location (**Table 6.3**). However the rate of evolutionary change between the four strains from the PPRV and CaPV was very high (**Figure 6.9**)

Table 6.3: Pairwise comparison showing percentage of identity between Capripox and *Peste- des-petits-ruminants* viruses.

	1	2	3	4
DRC_GPV_Mwenga_p32gene	-	99.75	47.17	48.40
DRC_GPV_Shabunda_p32gene	99.75	-	47.42	48.66
DRC_Mwenga_PPRV Fgene	47.17	47.42	-	96.95
DRC_Shabunda_PPRV Fgene	48.40	48.65	96.95	-

1=GPV_Mwenga_P32gene; 2= GPV_Shabunda_p32gene, 3=Mwenga_PPRV F gene; 4=Shabunda_PPRV F gene.
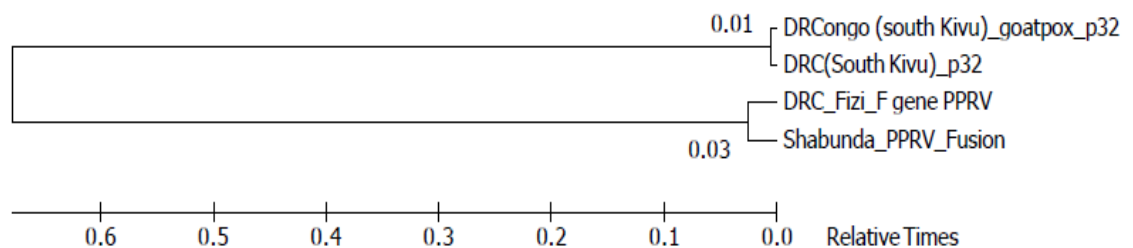
Figure 6.9: Phylogenetic relationship of P32 gene of Capripox virus and Fusion gene of *PPR* virus.

A Maximum-Likelihood phylogenetic tree showing the relationship of D R Congo CaPV and PPRV in Shabunda and Mwenga counties.

Further, a multiple alignment of the two PPR and the two CaP viruses showed several nucleotide substitutions as represented by stars in a portion fragment of 50 bp of the CaPV-*P32* gene aligned with PPRV-F gene from South Kivu (**Figure 6.10**).

Species/Abbrv	G1
1. DRCongo (south Kivu)_goatpox_p32	GCAGTAAAAGATTTTTTTTTAAAGATAAAAAAGATTATCATATAAGTTCC
2. DRC(South Kivu)_p32_goatpox	GCAGTAAAAGATTTTTTTTTAAAGATAAAAAAGATTATCATATAAGTTCC
3. DRC_Fizi_F gene PPRV	CCTGTCGTAGATCCTCTGTCAGTGATAACCAAGAATGTAAGACCAATTCA
4. Shabunda_PPRV_Fusion	CCTGTCGAAGATGCTCTGTCAGTGATAACCAAGAATGTAAGACCAATTCA

Figure 6.10: Multiple alignment of a 50 bp portion of the P32 gene of capripox and fusion gene of *PPR* viruses in South Kivu.

Where: **A**=Adenine; **C**=Cytosine; **G**=Guanine and **T**=Thymine.

Moreover, nucleotide sequences were translated into proteins using Mega 6 software by selecting a frame with no stop codons to check if the nucleotide sequence similarities affected the changes of amino acids in the aligned CaPV and PPRV protein sequences which are primary linear chains of proteins. Results of a portion of 24 amino acids of the 2 viruses aligned showed that several parts of the aligned protein sequences in the conserved regions showed the similarities (columns without a star including phenylalanine, tryptophan, valine, alanine and glycine) between the CaPV-*P32* gene and the PPRV-F gene proteins (**Figure 6.11**).

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Species/Abbrv	G		*		* * *	*		*	
1. DRCongo (south Kivu)_goatpox_p32		LSYKFLIWEKVAGGVVLQKFFWIM
2. DRC(South Kivu)_p32_goatpox		LSYKFLIWEKVAGGVVLQKFFWIM
3. DRC_Fizi_F gene PPRV		M*DQFKL*HPVAGPAV*PGLSWPE'
4. Shabunda_PPRV_Fusion		M*DQFRL*HPVAGPAVLPGLSWPE'

Figure 6.11: Multiple alignment of 24 bp protein sequences (amino acid) portion of the translated *P32* gene of capripox and fusion gene PPR viruses in South Kivu.

Where L (Leucine), M (Methionine), Y (Tyrosine), D (Aspartic Acid), K (Lysine),

Q (Glutamine), F (**Phenylalanine**),W (**Tryptophan**), E (Glutamic Acid), H (Histidine),

P (Proline), V (**Valine**), I (Isoleucine), A (**Alanine**), G (**Glycine**) and S (Serine),

6.4 DISCUSSION

Peste-des-Petits Ruminants and Capripox viruses are now considered as endemic and a

great threat towards the development of goat farming in D R Congo. Several previous

studies from Babiuk *et al.* (2008), Banyard *et al.* (2010), Esmaelizad *et al.* (2011) and

Venkatesan *et al.* (2014) reported on different aspects of PPRV and CaPV in goats in

several countries (Babiuk *et al.,* 2008; Banyard *et al.,* 2010; Esmaelizad *et al.,* 2011;

Venkatesan *et al.,* 2014). Babiuk *et al.* (2008) assessed the current distribution and

expansion of capripox viruses compared to past 50 years with recent SPP and GTP

outbreaks in Vietnam, Mongolia and Greece, and outbreaks of LSD in Ethiopia, Egypt

and Israel. The PPRV- CaPV co-infection prevalence of 38.7% (**Table 6.2**) obtained in

South Kivu was high and could easily explain the high mortality and morbidity rates

observed in this region. However a high prevalence of 84% was observed recently in clinical goats in India which was attributed to the occurrence of mixed infection of PPR and goatpox (Karim *et al.,* 2016). Rajak *et al.* (2005) identified that *Peste-des-petits-ruminants* was an immunosuppressive disease, and co-infection may elevate or increase the incidence and severity of associated diseases in small ruminant population. The factors that can explain the mixed CaPV and PPRV infection which was found in South Kivu still need further investigation. Saravanan *et al.* (2007), Kul *et al.* (2008), Mondal *et al.* (2009), Ozmen *et al.* (2009), Malik *et al.* (2011) and Karim *et al.* (2016) have also reported goatpox in a mixed infection with PPR. Settypalli *et al.* (2016), while investigating capripox virus, *Pasteurella multocida*, *Peste de petits ruminants* virus and *Mycoplasma capricolum* ssps (*capripneumoniae*) respectively in multiple infections using multiplex PCR, found prevalences of 98.31%, 95.48%, 102.77% and 91.46%, while, using singleplex PCR, found prevalences of 93.43%, 98.82%, 102.55% and 92.0%, respectively. Still, very little is well-known about the specific interactions that occur in concurrent infections (Malik *et al.,* 2011). This study found that the capripox virus that was circulating in South Kivu, D R Congo in co-infection with PPRV was lumpy skin disease virus (LSDV) as shown in **Figure 6.6**, **Figure 6.7** and **Figure 6.8**. This could be due to the communal farming system in the country where ruminants are reared together.

In the current study, El-Nahas *et al.* (2011) and Tuppurainen *et al.* (2017) proved that LSDV infects domestic cattle and Asian water buffalo in Egypt. However, Malik *et al.* (2011) confirmed that strains of LSDV may also replicate in sheep and goats in their

study on dual infection of PPR-GTP in central Indian indigenous goats. In cattle, LSDV outbreaks are related with high vector activity especially after the rains and mostly in the highlands (El-Nahas *et al.,* 2011). Many reports have shown that the LSD virus residence is unidentified during minimal or none vector activity (Tuppurainen *et al.*, 2017). Lamien *et al.* (20110 and Gelaye *et al.*(2013) were able to detect 250 copies and 20 copies of CaPV by using the classical PCR and real time PCR methods, respectively. Previous studies in India however, found the GTPV and SPPV (Santhamani *et al.,* 2014). However, only limited reports are available on molecular characterization of the LSDV for their exact lineage and host specificity. Wild ruminants including springbok, impala and giraffe are potentially susceptible to the virus (Lamien *et al.,* 2011; Tuppurainen *et al.*, 2017). Lumpy skin disease (LSD), sheeppox (SPP) and goatpox (GTP) diseases are categorized by the OIE as notifiable diseases due to their potential for rapid spread and substantial economic impact.

In this study, it was found that fusion gene of PPRV had some genetic relationship with the *P32* gene of capripox virus (**Table 6.3** and **Figure 6.9** and **6.10**) in goats in South Kivu. Due to the large genome size of the poxvirus, multiple genes or whole genome analysis is necessary to supplement the available molecular data. Several authors have confirmed that the *P32*, *GPCR* and *RPO30* genes carry specific molecular signatures for LSDV, SPPV and GTPV and can be used for differentiation of these viral species/lineages (LeGoffe *et al*., 2009; Lamien *et al.,* 2011). Differentiating the CaPVs

and PPRV seems to be important for an accurate and successful vaccination and control strategies of the two diseases.

In conclusion, the high multiple infection prevalence between *PPR* and capripox viruses detected and reported for the first time in the current study in South Kivu province in D R Congo including, Shabunda and Mwenga regions, warrants appropriate control measures since these viruses can be of threat to the livestock in the region. It is known that mobilizing livestock for vaccination is expensive for both the livestock owner and the agency providing vaccination. Therefore, a possibility of having a mixed combined PPR-CaP vaccine should be availed and addressed for proper control of the spread of these two diseases.

CHAPTER SEVEN: SOCIO-ECONOMIC IMPACT OF *PESTE DES PETITS RUMINANTS* IN SOUTH KIVU PROVINCE

7.1 INTRODUCTION

Peste des petits ruminants is a major sheep and goat economic disease also known as "cattle of the poor", affecting the pastoral herders in D.R. Congo and many other developing countries. It is an exceedingly transmissible virus-related infection, occurring primarily in epidemic proportions and can cause high mortality of up to 90% in immunologically naive sheep and goat populations, resulting in significant negative socio-economic impacts (Munir *et al.* 2013). *Peste-des-petits ruminants* is a major menace to the production of small ruminant and especially goats and sheep and it is ranked by pastoral communities among the top 10 diseases of small ruminants (Diallo, 2006). The economic losses of PPR outbreaks in Kenya during the outbreaks of 2006 and 2007 were estimated to have been over 15 million USD (Nyamweya *et al.*, 2009). After the successful global eradication of rinderpest which was a "PPR like disease" in 2011, there were calls for regional and global PPR eradication which supposed to start with the progressive control (Elsawalhy *et al.,* 2010; Baron *et al*., 2011; Anderson *et al*., 2011). In March 2015, OIE and FAO officially launched a new programme of PPR-Global eradication by 2030 (http://www.oie.int/eng/ppr2015/background.html) (OIE-FAO, 2015). Simultaneously, the same report had shown that *PPR* had severely affected sheep and goats in over 70 African, Middle East and parts of Asian countries and the disease had caused every year in regions that record more that 80% of the total goats and sheep of the world the loss from 1.5-2 billion USD. These affected regions have

more than 330 million of the total world's poorest people, depending mainly on the animals for their incomes and survival. Moreover, 50 other countries are at risk of been infected with PPR and only 48 countries in the whole world were formally recognized in May 2014 by the OIE as PPR-free areas (OIE-FAO, 2015). The valued recent expenses due vaccination against PPR varies between 270 and 380 Million USD per year. However, Jones *et al.* (2016) in their study on the analysis of benefit-cost economic impact of PPR eradication suggested strong economic returns from PPR eradication. Based on a 15-year programme with total discounted costs of 2.26 billion USD, they have estimated discounted benefits of 76.5 billion USD, yielding a net benefit of 74.2 billion USD (Jones *et al.,* 2016).

The annual direct loss due to *PPR*, i.e. dead goats & sheep value is estimated at 5.3 million USD (SADC, 2012b). Given that PPR has already been targeted by FAO and OIE as a major priority disease for global eradication, and D.R. Congo is one of the SADC countries where data on the economic impact due to PPR are not well documented despite several reported sporadic outbreaks, this study aimed at estimating the direct economic losses caused by PPR outbreaks based on perceived loss of benefits experienced by the South Kivu small ruminant holders from 2011-2016.

7.2 MATERIALS AND METHODS

7.2.1 Study area

This is as given in Chapter 3 section 3.2.1.

7.2.2 Sampling unit

The four regions (clusters) were selected purposively based on the current or previous PPR reports in that area. The cluster' sample size was determined by the proportional probability method using a formula proposed by Jost *et al.* (2010) and Catley *et al.* (2012):

$$\mathbf{n_{zone}} = \frac{Pi}{Pt} * N$$

Where; $\mathbf{n_{zone}}$= sample size per area; **Pi** is the number of farmers who had experienced the PPR in the past 5 years (2011-2016) and who kept both goats and sheep; **Pt** is the total population of small ruminant farmers in the whole study environment and **N** is the total sample size predetermined for the study. Moreover, due to accessibility of data and funds availability, the study was limited to 50-50-40-40 interviewees in Kalehe, Fizi, Mwenga and Shabunda, respectively and selected randomly, for the questionnaire survey (**Appendix 17**). Therefore, the study sample size consisted 180 farmers.

7.2.3 Data collection

The collection of data was adapted to participatory epidemiology (PE), and techniques for assessing the collection of epidemiological and socio-economic data of a disease, as described by several authors (Jost *et al.*, 2010; Catley *et al.*, 2012; Kihu *et al.*, 2015). Data were gathered from key informants through interviews and farmer group discussions guided by lists of open and closed questions pre-tested and adjusted prior to the start of the study. Animal value parameters were estimated from secondary data where the value of sheep and goats per unit of tropical livestock (TBU) were estimated

at 150 USD (Mude *et al.,* 2010). The model parameters collected in the field were the relative incidence, morbidity and mortality from PPR, herd structure by age groups and species. As for the administration of the questionnaire, in the field, the survey was carried out in the company of the technical staff of each village to facilitate both interpretations during interviews and sample collection where necessary; when animal(s) presented with clinical signs suggestive of PPR. The interview lasted for an average of 15 minutes. According to the language spoken by the breeder, the interview took place sometimes in Mashi, Kifuliro, Swahili, and / or in French with the help of local interpreters.

7.2.4 Economic evaluation method

The mortality and morbidity rate due to PPR was evaluated following the formula as suggested by Jost *et al.* (2010) and Catley *et al.* (2012):

$$\text{Mortality rate (\%)} = \frac{Number\ of\ dead\ animals\ (past\ 5\ years)}{Total\ number\ of\ animals\ possessed} \times 100$$

$$\text{Morbidity rate (\%)} = \frac{Number\ of\ survived\ animals\ with\ low\ economic\ value}{Total\ number\ of\ animals\ possessed} \times 100$$

In addition, the morbidity was calculated by considering the number of animals that had been infected with PPR in the past 5 years (from 2011-2016), with less or no economic value but not dead. The evaluation of the economic impact of PPR was carried out through two strategies: Strategy A and Strategy B. Strategy A reflected the cost of

disease without control, while Strategy B reflected the cost of disease in the presence of control.

7.2.4.1 Strategy A

Strategy **A** assayed direct and indirect costs of the disease. Direct costs were those related to mortality or morbidity.

The direct costs related to mortality and included: the number of dead animals by age group **(n),** the average selling price of an animal **(pa)**. The monetary value of mortality losses, M, was the product of these two parameters and it was calculated using the following formula:

$$M = n \times pa.$$

The direct cost related to morbidity was characterized here by the weight losses linked to the disease but also to the abortions. Considering the weight losses, these costs included the estimated average loss of weight per animal expressed in kg (**na**), the number of animals that have lost weight (**N**), the average selling price of kg to market (**p**). The calculation of direct costs related to morbidity by weight loss, **P,** was therefore:

$$P = N * na * p$$

Considering morbidity due to abortions, these costs included the number of females having aborted (**Na**), the cost of abortion (**c**), generally estimated from the cost of a lamb / kid. The calculation of direct costs related to morbidity due to abortions, **A,** was therefore;

$$A = Na*c$$

Thus the direct cost associated with morbidity, M', was calculated using the following summary formula;

$$M'= P + A$$

The indirect costs were all negative impacts of PPR, other than mortality and production losses. These costs were not taken into account as they are difficult to quantify.

7.2.4.2 Strategy B

Strategy B, which assessed losses under control conditions, was equivalent to the costs of disease despite the control; not estimating the costs of the control.

7.2.5 Data analysis

Raw data collected during the survey were scanned and then encoded in the Excel spreadsheet. The analysis of the data consisted a statistical evaluation of the information collected. It was done with XLSTAT and STATIX software. Apart from the descriptive statistics, the statistical relationship between quantitative parameters was determined by KHI TWO test. The ANOVA test was used to check whether certain quantitative parameters varied significantly according to the study zones and species.

7.3 RESULTS

7.3.1 Trade of goats and sheep in South Kivu

It was found that 99.5% of investigated farmers were selling their animals as per the financial need of the household. The selling periods varied, but in general, 80.3% of

farmers confirmed that animals were sold throughout the year (p-value> 0.05). Only 11.3% of farmers were selling the animals strictly during the annual start of school, which event occurred usually in September every year and 8.06% of interviewees were selling their animals only during the holidays. The animals sold were paid-for in cash (98.8%). Moreover, it was found that animals were sold in most cases to the market located far away (55.8%). Twenty eight percent (28.0%) of farmers brought their animals to near markets within the same village and only 15.6% of farmers were selling the animals from home. A total of 99.5% of farmers in the study area confirmed that animals were sold and the prices were determined based on the animal's weight, sex and age. The difference between all the evaluated parameters regarding the sale of animals and their specific levels were not significant (p-value>0.05) (**Table 7.1**).

Table 7.1: Trade of small ruminants (Goats and Sheep) in South Kivu

Parameters	Levels	South Kivu				Mean	χ2	*p-value*
		Kalehe (n=50)	Fizi (n=50)	Mwenga (n=40)	Shabunda (n=40)			
Animal selling objectives	*No*	0	2	0	0	0.5	2.6	0.46
	Yes	100	98	100	100	99.5		
Animal Selling period	*NA*	0	2.0	0.0	0.00	0.5	9.4	0.40
	Holiday	6	10.0	5.9	10.34	8.1		
	Starting school	10	4.0	9.8	20.69	11.1		
	Whole year	84	84.0	84.3	68.96	80.3		
Selling purpose	*NA*	0	2	0	0	0.5	2.6	0.45
	Need of money	100	98	100	100	99.5		
Terms of sale	*Paid in debts*	0	5	0	0	1.3	2.6	0.46
	Paid in cash	100	95	100	100	98.8		
Selling place	*NA*	0	2	0	0	0.5	6.3	0.71
	Home	12	14	19	17.2	15.6		
	Near Market	26	34	31.4	20.7	28.0		
	Market far	62	50	49.0	62.1	55.8		
Selling methods	*NA*	0	2	0	0	0.5	-	-
	Based on weight and sex	100	98	100	100	99.5		

7.3.2 Economic characteristics of goats and sheep

7.3.2.1 Selling price of goats and sheep

The results showed that there were few animals (1.7 ± 0.8) sold per year during the last 5 years. The selling price was valued at an average of 52.2 USD. Variation in the selling prices of the animals was observed as the sale took place at the various animals' physiological state (pregnancy or not). The average minimum selling price was 27.8 USD; the average being 36.5 USD and the maximum selling price was 63 USD (**Table 7.2**).

Table 7.2: Selling price of goats and sheep in South Kivu (in USD)

Regions	Animals solde annually	Selling price in market	Minimum selling price	Average Selling price	Maximum selling price
Kalehe (n=50)	1.5±0.6	52.2±8.3	27.9±5.4	37.8±5.4	63.6±6.2
Fizi (n=50)	1.7±0.8	49.1±10.2	27.02±6.3	34.7±8.5	61.4±14.1
Mwenga (n=40)	1.9±0.8	51.8±7.1	28.4±4.0	36.8±6.4	62.8±5.6
Shabunda (n=40)	1.8±0.8	55.5±7.6	27.9±4.5	36.7±5.5	64.3±4.7
Gen. Mean	**1.7±0.8**	**52.2±8.3**	**27.8±5.1**	**36.5±6.5**	**63.0±7.7**

USD: *United States of American dollars*

7.3.2.2 Direct and indirect economic losses due PPR

The average mortality rate of small ruminants observed during the last 5 years (2011-2016) due to suspicion of PPR was high in goats (60.4 and 41.6%, respectively) in animals aged above 1 year compared to animals of less than 1 year old. Similarly, the morbidity rate was estimated high in goats of more than 1 year old (76.3%) compared to goats of less than a year (22.7%). Mortality and morbidity rates in sheep aged above 1 year were 42.5 and 23.5%, respectively, while it was 18 and 20.5% for sheep of less than a year (**Table 7.3**).

Table 7.3: Cumulative Mortality and Morbidity rates of PPR-suspected goats and sheeps

Species	Mortality rate (%)		Morbidity rate (%)	
	Old animals (>1year)	Young animals (<1year)	Old animals (>1year)	Young animals (<1year)
Sheep	42.5	18	23.5	20.5
Goats	60.4	41.6	76.3	22.7

The high morbidity rate due to suspicion of PPR was observed in old animals in sheep (0.28±0.64) compared to the young ones (0.22±0.61) in Fizi region. It was 0.4±0.1 in Shabunda region. However, the high mortality rate due to suspicion of PPR was registered in young goats 3.14±1.7 compared to the old animals 2.24±1.10 in Shabunda region (**Table 7.4**). The weight loss associated with the disease was on an average of 3 kg per animal, with an average of 3.28 animals affected per farm. Animals lost more weight in Mwenga and Shabunda regions with respectively 3.1±0.9Kgs and 3.4±0.9

Kgs compared to Fizi and Kalehe with 2.9±0.1 and 2.6±1.4 Kgs, respectively, but these differences were not significant (p>0.05). The average price of kids /lambs varied from 20.4 to 25.1 USD. It was moreover observed that 1kg of goat/sheep meat was sold at an average of 3110.4±345.1Congolese franc (FC) (1USD equivalent of 1500 Congolese Franc). The average price per kilo of meat at the market was around an average of 3110.4 Congolese francs (2 USD). Very few cases of abortion were observed (0.7±0.8) in South Kivu in PPR suspected animals (**Table 7.5**).

Table 7.4: Estimation of mortality and morbidity rates in goats and sheep due to PPR suspicion

Region	Old Sheep	Young Sheep	Old Goats	Young Goats	Mobidity		Mortality	
					Old Sheep	Young Sheep	Old Goats	Young Goats
Kalehe	0.38±0.7	0.94±1.68	1.52±1.30	5.22±3.02	0.26±0.52	0.10±0.30	0.86±0.92	0.84±1.15
Fizi	0.32±0.8	0.80±1.77	1.7±2,77	5.18±2.77	0.28±0.64	0.22±0.61	1.22±1.01	1.18±1.05
Mwenga	0.12±0.4	0.45±0.97	3.88±1.75	9.96±4.86	0.39±0.19	0.12±0.32	1.6±1.10	2.08±1.40
Shabunda	0.31±0.6	0.76±1.22	5.3±1.73	11.97±4.11	0.10±0.30	0.11±0.40	2.24±1.10	3.14±1.72
Gen. Mean	**0.28±0.63**	**0.74±1.41**	**3.10±1.89**	**8.08±3.69**	**0.17±0.41**	**0.14±0.41**	**1.48±1.03**	**1.81±1.33**

Old sheep/goat :> 12months, young sheep/goats: <12months

Table 7.5: Evaluation of morbidity parameters due to PPR suspicion in goats and sheep in South Kivu

Region	Weight loss (Kg)	Animals loss weight	Selling price/kg of meat (FC)	Number of abortion	Selling price of kids (USD)
Kalehe	2.59±1.4	1.4±1.2	3192±374.6	0.44±0.64	22.6±2.498
Fizi	2.90±0.99	3.10±1.81	3240±349.9	0.84±0.88	24.6±3.72
Mwenga	3.08±0.91	3.43±2.9	3078.4±318.9	0.81±0.79	22.7±2.50
Shabunda	3.42±0.85	5.20±1.89	2931.3±340.5	0.69±0.84	22.76±2.5
Gen. Mean	**3±1.04***pv=0.079*	**3.28±1.95**	**3110.4±345.98**	**0.7±0.79**	**23.17±2.81**

FC (Congolese franc): 1USD=1600FC

The results showed that there was a significant difference between mortality of sheep in the different regions (Mwenga, Shabunda, Fizi and Kalehe) (p<0.05. The mortality was high in Mwenga and Shabunda compared Fizi and Kalehe (**Table 7.6**). However, any significant difference between the goat mortality rates was observed in the different surveilled regions (p> 0.05) (**Table 7.7**). There was a significant difference of sheep mortality rate in different age classes (p<0.05) (**Table 7.8**).The high mortality rate was observed in older sheep (over 12 months) compared to younger animals (<12 months). Similarly, there was a significant difference of goat mortality rate in different age groups (p<0.05) (**Table 7.9**).The mortality rate was high in old animals (>12 months) compared to animals of less than 12 months old.

Table 7.6: Analysis of variance of sheep mortality rates in the studied regions

Source	DF	Sum of Squares	Mean Squares	F	Pr>F
Treatment	1	0.573	0.573	9.731	0.002
Error	358	21.063	0.059		
Total	359	21.636			

DF: Degree of freedom

Table 7.7: Analysis of variance of goat mortality rates in the studied regions

Source	DF	Sum of Squares	Mean Squares	F	Pr > F
Treatment	1	1.650	1.650	3.495	0.062
Error	357	168.577	0.472		
Total	358	170.227			

DF: Degree of freedom

Table 7.8: Analysis of variance of sheep mortality rates in different ages of animals.

Source	DF	Sum of Squares	Mean Squares	F	Pr > F
Traitement	1	0.320	0.320	5.373	0.021

Error	358	21.316	0.060
Total	359	21.636	

DF: Degree of freedom

Table 7.9: Analysis of variance of goat mortality rates in different ages of animals.

Source	DF	Sum Squares	Mean Squares	F	Pr > F
Treatment	1	5.319	5.319	11.514	0.001
Error	357	164.908	0.462		
Total	358	170.227			

DF: Degree of freedom

7.3.2.3 Estimated loss due to mortality and morbidity

The price of the animals in the market depended on their breed, the physio ecological status, age and health status. Results showed that the economic loss due to the mortality rate of animals suspected with PPR was approximately 10.3 USD and 120.7 USD for sheep and goats respectively (**Table 7.10**). Socio-economic losses associated with PPR were influenced by the morbidity rate as well as the mortality rate. Mortality and morbidity rates due to weight losses were 33.661.7 Congolese francs (equivalent to 21.1 USD) (**Table 7.11**). The loss associated with morbidity due to abortions varied among species. Low costs were found for sheep compared to goat species at 9.8 and 16.6 USD, respectively (**Table 7.12**). Finally, it was observed that the economic losses due to PPR associated with morbidity rate were low in sheep (48 293.7 Congolese francs; equivalent to 30.2 USD) compared to goats (59269.7 Congolese francs; equivalent to 37.1 USD), where 1500 Congolese francs (FC) equals 1 USD (**Table 7.13**).

Table 7.10: Economic loss due to mortality (M) of PPR-suspected goats and sheep

Species	Age group	n	Pa	M=(n*pa)
Sheep	Old(>1year)	0.17	20.8	3.536
	Young (<1year)	0.14	48	6.72
	Total	**0.31**	**68.8**	**10.256**
Goat	Old(>1year)	1.14	23.1	26.3915
	Young (<1year)	1.81	52.15	94.3915
	Total	**2.95**	**75.25**	**120.725,5**

n: number of dead animals by age group; Pa: average selling price of an animal

Table 7.11: Costs related to Morbidity (P) due to weight loss (in CF).

Species	Age group	na	N	p	P= (na*N*p)
Sheep	Old (>1year)	3,3	2,99	3310,2	32661,7434
	Young (<1year)	3,3	2,99	3310,2	32661,7434
	Total	**3,3**	**2,99**	**3310,2**	**32661,7434**
Goat	Old (>1year)	3,3	2,99	3310,2	32661,7434
	Young (<1year)	3,3	2,99	3310,2	32661,7434
	Total	**3,3**	**2,99**	**3310,2**	**32661,7434**

na: average loss of weight per animal expressed in kg; N number of animals that have lost weight; p:average selling price of kg to market; CF: Congolese Franc.

Table 7.12: Costs related to morbidity (A) due to Abortions.

Species	Na	C	A= (Na*C)
Sheep	0,47	20,8	**9,776**
Goats	0,69	24,1	**16,629**

Na: number of females having aborted; C: Cost of abortion

Table 7.13: Total direct cost due to morbidity (M') associated with PPR

Species	Formula	Losses (cost)
Sheep	$M_1' = P_1 + A_1$	$M_1' = 32661,74 + 9,77 * 1600Fc =$ **48293,74Fc**
Goats	$M_2' = P_2 + A_2$	$M_2' = 32661,74 + 16,63 * 1600Fc =$ **59269,74Fc**

Fc: Congolese franc; M: Cost due to morbidity

7.4 DISCUSSION

Small ruminants, espencially goats and sheep considered as "mobile banks"; also referred to as "poor man's cow", are sources of income for many African farmers. They can easily and quickly be mobilized to acquire money for household expenditures. It was found that 99.5% of investigated farmers were selling their goats and sheep due to financial need of the household **(Table 7.1)**. The selling price depended on many factors such as animal weight, health status, race, age and sex. Over 80.32% of farmers interviewed in South Kivu confirmed that animals were sold throughout the year. The frequency of selling increased during festivities, dowries, cultural and religious ceremonies. Report from FAO (2009a) showed the cultural and socio-economic roles that goats and sheep are playing in religious and traditional events. Elsawalhy *et al*. (2010) demonstrated that goats and sheep provided a high social status to individuals and households and also served as the much envied symbol of wealth and respect among pastoral communities. In D.R. Congo it was observed that goats and sheep were more readily marketed than large ruminants and were often slaughtered for home consumption, as large ruminants are too much for a family; most of it will remain and get spoilt; these large animals like cattle are normally utilized in big celebrations and for dowry. The average selling price for a healthy

mature goat was valued at 52.2 USD. However, this price could be reduced up to 27.8 USD if the animal is not healthy; weight being very critical. Specific few cases where goats were sold at 63 USD were recorded, as referred to in Table 7.2. A registration transfer contract must be signed and shared when a seller of a registered animal want to sell it. The contract must have the name of new owner within six months according to the Animal Pedigree Act (APC).

Results of the current study showed that, during the past five years (from 2011-2016), the average goat mortality rate as a result of PPR was 60.4 % and morbidity rate was 76.3%; those for sheep were 42.5 and 18%, respectively (**Table 7.3**). However, these high mortality and morbidity rates found could also be explained by factors other than PPR, including: farm management, breeds, nutrition. Rossiter (2004) reported that morbidity and mortality rates from PPR could go up to 90 and 100% in goats and sheep, respectively. De Nardi *et al.* (2012) reported mortality and morbidity rates of up to 99% in the outbreak in Algeria. Dundon *et al.* (2015) found morbidity and mortality rates of 80% in Kenya. However this rate increased in cases of mixed infection. Several authors confirmed the high mortality and morbidity rates in goats compared to sheep (Zhao *et al.*, 2010). The middle form of the disease in sheep might be due to their natural immunity. In endemic countries like Somalia and Cote d'Ivoire, normal morbidity rates ranged from 6.2 to 65% and 48.4 to 56.6%, respectively. During epidemics these rates rose to between 86 to 100%; similar ranges have been reported in Kenya, Ethiopia and Eritrea. Mortality rates also varied with reports ranging between 0-97% in Cote d'Ivoire; 69 to 74% in Tanzania; 33 to 90% in Kenya, Ethiopia and Eritrea. However, previous studies done by Diallo (2006), Nyamweya *et al.* (2009), Elsawalhy *et al.* (2010) and Kihu *et al.* (2015)

showed that the establishment of the rates depended on methodology used in data collection, species and farming systems studied.

An average weight loss associated with the disease was estimated at 3 kg for each animal during early days of infection. This could increase with animal susceptibility to PPR but also the duration of the infection cycle. Following PPRV infection, the incubation period varied from 3-4 days (Lefèvre and Diallo, 1990). Bailey *et al*., 2005 confirmed that the PPR virus replication occurs during this stage in the draining lymph nodes. It was moreover observed that 1kg of goat/sheep meat was sold at an average of 3110. 4±345.98 FC (approximatively 3 USD), this price could decrease if a diseased animal lost about 3kg; which happens within few days after infection or during incubation period; the weight loss being due to anorexia (lack of appetite) which animals develop when they are sick. Rushton *et al*. (1999) and Rushton (2002; 2009) listed the visible losses experienced due to PPR as: dead animals, thin animals, poorly developed animals, low returns and poor quality products. However, the delay in selling animals and their derived products, problems of fertility, costs related to public health, high prices of livestock and their products and heard structure changes can be classified to invisible losses. Other additional losses include: buying of medicines, vaccines, insecticides; time lost while attending to the sick animals; and payment towards veterinary services. Also, access to better markets is denied or reduced.

The mortality and morbidity rates due to PPR were high in old animals compared to young ones because the young animals may have been protected by maternal antibodies. Moreover, the high goat mortality and morbidity rates compared to sheep as referred in **Tables 7.6; 7.7; 7.8** and **7.9** could also be attributed to their higher susceptibly to the disease compared to sheep; this

study's serological results presented in Chapter 3 explains this fact. Moreover, the high

mortality and morbidity rates observed in Shabunda and Mwenga regions could be explained

by the high number of sporadic PPR outbreaks that have been registered in these regions

compared to the other regions (IPAPEL, 2016; FAO, 2016a). Khalafalla *et al.* (2010) and Perry

et al. (2002) ranked PPR in the top ten diseases of small ruminants. The disease has also been

ranked by pastoral communities among the top ten small ruminant diseases (Diallo, 2006).

In this study, it was observed that, during a PPR outbreak, the economic losses were about 30.2

USD in sheep and 37.1USD in goats per herd per day (**Table 7.13**); the losses being higher in

cases where the outbreak lasted for longer period in a farm;   indirect costs not considered. From

its emergence in the D.R. Congo between 2010-2012, a total of 120, 000 goats & sheep died

due to PPR infection in two years, with losses valued at 5.3 million USD (SADC, 2012b;

IPAPEL, 2016). In India, the economic losses associated to PPR were valued at 39 million USD

(Chauhan *et al.,* 2009). Moreover, in their previous study in Nigeria, Hamdy *et al.* (1976)

reported around 1.5 Million USD as losses induced by PPR. Few years later, Stem (1993)

indicated that vaccination against PPR in Niger returned the benefits of 24 million USD in five

years after two million USD investments. It was estimated a direct losses every of 1.2 to 1.7

billion USD due to PPR in the OIE-FAO Global PPR eradication program with a total amount

of 270 and 380 million USD valued as current costs for PPR vaccination. A total ranging from

1.45 and 2.1 billion USD per year is consider the losses impact due to PPR alone (FAO, 2015).

In Tanzania it was estimated that 330,910 kids/lambs were not borne due to abortions. In Kenya

and Tanzania 10% of households lost their entire herd or flock. It was estimated that in Kenya,

Tanzania and Somalia, milk production losses were in the region of 2 million litres (Rushton,

2009). Previous studies in India indicated the losses of Rs 523 (8.44 USD) in Madhya Pradesh due to mortality rates associated with PPR (Awase *et al*, 2013) and Rs 918 (14.81 USD) in sheep and Rs 945 (15.24 USD) in goats in Maharastra (Thombare and Sinha, 2009).

In conclusion, the South Kivu agro-pastoralist community recognises PPR as a major economic disease affecting goats and sheep with the daily economic losses associated with morbidity and mortality rates of approximately 30.2 USD for sheep and 37.05 USD for goats; and 10.26 USD for sheep and 120.72 USD for goats, respectively.

CHAPTER EIGHT: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

8.1 GENERAL DISCUSSION

8.1.1 PPRV seroprevalence and associated risk factors

Since its report in 2008 in Democratic Republic of the Congo, the current research has established for the first time the spatial distribution of *Peste-des-petits ruminants* virus showing high seroprevalence (53.4% in goats and 21.3% in sheep) in South Kivu region in Eastern of D.R. Congo. There is no previous data for the study regions to compare with, but previous reports from Uganda showed an overall PPRV sero-prevalence of 57.6 % (CI = 95%, 48.8 – 66.4%) after testing 280 goats (Mulindwa *et al.,* 2011) while Kgotlele *et al.* (2014) reported a sero-prevalence of 24.3% in Tanzania, Swai *et al*. (2009). Moreover, Muse *et al*. (2012) found seroprevalences of 45.4% and 31.0%, respectively, in Northern and Southern Tanzania, showing high susceptibility in sheep compared to goats. *Peste des petits ruminants* serological survey in Tanzania by Kivaria *et al.* (2013) also demonstrated higher susceptibility in sheep; the overall sero-prevalence was 22.10% (68.0% in goats and 73.0% in sheep).

In Algeria, an overall PPRV sero-prevalence of 30.45% was recorded with a mean of 29.87% ± 2.11 (Kardjadj *et al.,* 2015), while different studies conducted in Sudan, showed high levels of PPRV seroprevalence including 50.6%, 62.8% and 61.8%, respectively (Abubakar *et al.,* 2009). In Kenya, Kihu *et al.* (2015) found a higher PPRV sero-prevalence of 40% in goats compared to 32% in sheep. Ozkul *et al.* (2002) reported a seroprevalence of 45.4% in Turkey (Asia) and 31.0% in in Central Asia, Tajikistan (Kwiatek *et al*., 2007). Sarker and Islam (2011) found a prevalence of 20.57% (n=129) in goats of Rajshahi, Bangladesh. *Peste des petits ruminants* (PPR) associated determinants found in South Kivu included agro-ecologic location, species,

breeds, sex, age, farming and grazing system, watering source, herd composition and size, wind-speed and farm-to-farm animal exchange. Other studies have also reported host determinant factors of PPR spread, among which were age of the animal, species, breed and sex (Munir *et al*, 2013). Singh *et al.* (2004b) showed the high and at early age-stage off-take of male small stock for social economic activities compared to females which are kept longer in the herds for productive purposes. Luka *et al.* (2011) found that young animals are more predisposed to PPR virus infection because they are less likely to have developed defensive PPRV antibody titers. The differences in the seroprevalence of antibodies to PPRV and associated risk factors could be attributed to differences in management system of small ruminants, levels of natural immunity, variable natural PPRV infection rates in different geographical areas, sensitivity of diagnostic tests used, sampling procedures used or technical knowledge (Singh *et al.*, 2004).

8.1.2 Molecular prevalence and genetic characterization of PPRV

The prevalence of 64% found, in this study, using molecular based technique (RT-PCR) was higher compared to the serological prevalence of 45.3% found using competitive ELISA. This is due to the high specificity and sensitivity of PCR compared to the immunological assay used (cELISA). Moreover, samples for PPRV antibody prevalence were collected randomly from unvaccinated and asymptomatic animals while samples for virus isolation were collected purposively from PPR-suspected animals. Previous studies done in Tanzania have detected high PPRV prevalence range of 29.6 - 31.1% in the goats tested (Muse *et al.,* 2012; Kivaria *et al.,* 2013; Kgotlele *et al.*, 2014).

Focused on partial nucleoprotein (NP), fusion (F) and haemagglutinin (H) gene sequences it was found that only lineage III of PPRV was circulating in Mwenga, Shabunda and Kalehe counties in South Kivu of D.R. Congo. Knowing that PPRV is a transboundary disease, this lineage might

have been introduced through animal trade market from East Africa, including Tanzania, Uganda and Kenya. Several studies including Swai *et al*. (2009), Karimuribo *et al*. (2011), Luka *et al*. (2012b), Kivaria *et al*. (2013), Lembo *et al.* (2013) and Dundon *et al.* (2015) reported the circulation of PPRV of Lineage III in East Africa.

8.1.3 Mixed infection of PPR and Capripox viruses

A co-infection prevalence of 38.7% was obtained from this study; it included co-infection of PPR and CaP viruses, especially lumpy skin disease. Although the distribution and prevalence of capripox viruses had considerably decreased in the past 50 years, in D.R. Congo, they are now expanding their territory (Babiuk *et al.,* 2008). Rajak *et al.* (2005) identified that *Peste des petits ruminants* was an immunosuppressive disease, and co-infection may elevate or increase the incidence and severity of associated diseases in small ruminant population. Previously Saravanan *et al.* (2007), Kul *et al.* (2008), Mondal *et al.* (2009), Ozmen *et al.* (2009), Malik *et al.* (2011) and Karim *et al*. (2016) reported also goatpox in a mixed infection with PPR but still, very little is known about the specific interactions that occur in concurrent infections (Malik *et al.,* 2011), hence factors that can explain the mixed CaPV and PPRV infections found in South Kivu need to be investigated. The presence of LSD virus (LSDV) in dual infection with PPRV could be due to the communal farming system in the country where ruminants are reared together knowing that LSDV is more common in cattle rather than in small ruminants, as confirmed by El-Nahas *et al.* (2011) and Tuppurainen *et al.* (2017). In their study on co-infection between PPRV-GPV, Malik *et al.* (2011) confirmed that strains of LSDV may also replicate in sheep and goats.

8.1.4 Genetic diversity of mtDNA (*d-loop*) of goats suspected with PPR

Based on *d-loop* mtDNA control region sequence of goats suspected and confirmed to be infected with PPRV, two maternal haplogroups (A and B) were found. The high genetic diversity was discovered among 56 haplotypes in 1,220-bp sequences of the 111 sequenced goats. The goats studied clustered into three goat gene pools including Shabunda-Mwenga, Kalehe and Fizi. Around 99% of the studied goats which were confirmed with PPRV belonged to lineage A and only 1% clustered in lineage B group. Naderi *et al.* (2007), showed that haplogroup B was more dominant in Southern African countries, while haplogroup A was widely distributed with high frequencies found in North and Latin America. Several authors among them Luikart *et al.* (2001), Sultana *et al.* (2003), Chen *et al.* (2005) and Naderi *et al.* (2007) found multiple maternal haplogroups of domestic goat that were clustered into six different haplotypes/ lineages (A, B, C, D, F and G) with lineage A having a high level of genetic diversity compared to B but also are more susceptible to disease.

This could partially explain the susceptibility to PPR of indigenous goat populations in South Kivu which are originating from lineages genetically susceptible. However, there is need for more research on comparative genomics to identify and characterize SNPs associated with disease resistance in host. A total of 10.7 nucleotide differences, 120 segregating sites and 124 mutations were detected in the studied goats, with 83.2% of the total genetic variation described by genetic variation between individuals from the studied populations, 11.2% among groups and only 5.6% of the variation was attributed to genetic differences between populations. Similar results were reported in three goat populations in Morocco, whereby 64 polymorphic sites were detected and 40 haplotypes belonged to lineage A (Benjelloun *et al.,* 2015). In the whole mitochondrial genome sequence analysis, Colli *et al.* (2015) also discovered 229 polymorphic

sites in goat matrilineal variability. This estimation is similar to previous fundings where 83% of the variation within population were observed in Indian goats, but differed from South and Central Asian goats where 69% of variation was observed within population, and for European, African and Asian goats where 78.7% of variation was observed within population (Amills *et al.,* 2004; Amills *et al.,* 2008). Kalehe goat's population showed relatively higher differentiation and lower estimates (0.873±0.051) of nucleotide diversity compared to the rest of the studied animals (**Figure 5.3, Figure 5.4 and Table 5.3**). This could be due to the minimal gene flow level that occurs in Kalehe region and inadaptability of goats in Kalehe area compared to the rest part of South Kivu. The bimodal distribution presented in **Fig. 5.4** showed the existence of one major and one minor event of population expansions in D.R. Congo goat populations in the past. Moreover, the positive and non-significant Fs values obtained in this study confirmed a slow population expansion (**Table 5.4**). Previously, Chen *et al.* (2005) observed a negative Fs estimate (Fs = -23.57<0.01) in goats from China. However, human socio-economics and cultural connections could explain the demographic expansion of goat's population. Moreover recently, curves of multimodal mismatch were found in Anatolian Black and Angora populations (Akis *et al.,* 2014).

8.1.5 Socio-economic impact of *Peste de petit ruminants* in South Kivu

Goats and sheep are more readily marketed than large ruminants and are often slaughtered for home consumption, as large ruminants are too much for a family; most of it will remain and get spoilt. About 80.3% of the interviewed farmers in South Kivu confirmed that animals were sold throughout the year and the frequency of selling increased more during festivities, dowries, cultural and religious events. Report from FAO (2009a) showed the socio-cultural importance of sheep and goats despite the socio-economic role that they are playing. Goats and sheep are used

as gifts during dowry ceremonies and as symbols for traditional and religious ceremonials. Following the PPR outbreaks onset in South Kivu province since in 2008, the disease has triggered relatively large economic losses to South Kivu pastoral herders. This has significantly reduced the animal trade and increased the selling price of healthy animals.

The mature healthy goat costed 52.2USD on average. In cases where the animal was sick, like in the case on PPR infection, or lost a lot of weight the price decreased up to 27.8 USD. It was found from 2011-2016, the mortality and morbidity rates due to PPR suspicion in South Kivu was 60.4 and 76.3% respectively in goat and 42.5 and 18% in sheep (Table 7.3). Previous studies in Kenya for instance have reported a high equal mortality and morbidity rate of 80% in goats (Dundon *et al.,* 2015). Moreover, previous studies from Diallo (2006), Nyamweya *et al.* (2009), Elsawalhy *et al.* (2010) and Kihu *et al.* (2015) have reported morbidity rate ranging between 6.2% and 65% in Somalia, 48.4-56% in Ivory Coast, 69-74 in Tanzania and 33-90% in Kenya respectively. The differences observed in these rates might be due to differences in data collection methods, farming system, environmental differences. The daily losses due to PPR of 30.2 USD per farm during a PPR outbreak was estimated and was attributable to morbidity rates in sheep and 37.1 USD of farmer daily losses in goats due to the mortality rates. Approximatively 10.3 USD daily losses for sheep and 120.7 USD for goats were observed daily due to mortality rates in a PPR outbreak.

The Southern African Development Community (SADC) report in 2012 showed that since the first report of PPR in D.R. Congo from 2010 to 2012, at total of 120,000 goats and/or sheep have died due to PPR and an estimation of around one million and 600,000 small ruminants, goats and sheep respectively (representing one-quarter of goats and two-thirds of sheep of the whole country) are at risk of PPR infection. The annual direct loss due to *PPR,* i.e. dead goat & sheep

value, was therefore estimated at 5.3 million USD in D.R. Congo (SADC, 2012b). Chauhan *et al.* (2009), found en financial loss due to PPR in India of 39 million USD each year. A previous study conducted by Hamdy *et al.* (1976) in Nigeria reported losses induced by PPR of 1.5 Million USD and recently Kenya shillings 11.1 billion (equivalent to 10 million USD) in Turkana county in Kenya (Kihu *et al.*, 2015). Moreover, the OIE-FAO Global PPR eradication program estimated the direct annual losses to be between 1.2 and 1.7 USD billion (OIE-FAO, 2015). Without appropriate coordinated plan, there is alarm that

PPRV will extent to neighboring countries including Rwanda, Tanzania and Burundi and some other areas within the country that have not experienced the disease yet (North Kivu, Maniema and Katanga provinces).

8.2 CONCLUSIONS

1. *Peste des petits ruminants* outbreaks occur in Eastern of D.R. Congo, in South Kivu region, causing high mortality rates in indigenous goats and sheep. Molecular based techniques used in this study such as RT-PCR, cELISA and cytopathic effects (CPE) on VERO cells confirmed the high PPRV prevalence in South Kivu.

2. The spatial distribution and high PPRV seroprevalence found in unvaccinated goats and sheep in D.R. Congo indicated that PPRV is circulating resulting in the detection of natural PPRV-specific antibodies.

3. Several risk factors were associated with PPRV sero-status in D.R. Congo including spatial and farm management (geographic location, species, breeds, sex, watering source, wind-speed, age, heard size and composition, grazing system and farm-to-farm animal exchange). High PPR risk areas in South Kivu were identified and mapped.

4. *Peste des petits ruminants* virus obtained from Eastern D.R. Congo clustered genetically with PPRV strains of Lineage III from East Africa, including Tanzania, Uganda and Kenya which differed to the lineage found previously in Western part of the country. Several mutations showed by mismatch at both nucleotide and protein sequence levels were observed between South Kivu PPRV strains found in this study and the Nigeria 75 vaccine used in and across the country. This study has demonstrated the transboundary importance of this disease in the region.

5. Lumpy Skin Disease (LSD) of capripox virus (CaPV) was found in coinfection with PPR virus giving there constraint for diagnostics.

6. High levels of intrapopulation diversity and the weak phylogeographic structuring at the *D-loop* of mitochondrial DNA found in indigenous goats in South Kivu proved the existence of strong gene flow in goat populations, probably due to broad trans-border goat's movement in the past, which could explain the spread of PPRV across the country.

7. South Kivu pastoralist community recognises PPR as a major economic disease affecting the goats and sheep and it has the potential of disrupting cultural set up and local economy with the economic losses due to morbidity rate of approximately 30.2 USD or sheep and 37.05 USD for goats and mortality rate of approximately 10.26 USD for sheep and 120.72 USD for goats per day during a fresh PPR outbreak.

8.3 RECOMMENDATIONS

- Since this is the first seroprevalence study in the South Kivu region, mass vaccination is recommended with reagrds to the high risk areas that we have found and mapped, with no discrimination of sex, age and origin of animals, in order to protect the animals from PPR infection. Hower this mass vaccination should also consider the non infected area for prevention and protected immunity.

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- The development of locally-adapted vaccines that will be compatible to the PPRV strains to improve the protection and control of the disease is suggested,

- There is need to incorporate indigenous knowledge on livestock diseases for purposes of understanding the various diseases within the communities, through setting up of strong participatory surveillance systems that would involve the communities as the basic elements of disease surveillance intelligence gathering.

- Epidemiological survey of *peste des petits ruminnats* should emphasize on the regional approach and not restricted as PPR is a Trans-Boundary Disease

- A strategic trans-border movement control of animals and feeds is required in South Kivu region in order to reduce the spread from infected areas to non-infected regions which have never reported a PPR outbreak. However, since the disease was confirmed in neighboring zones, a quick animal screening in PPR-free areas is required to ascertain their status. In-depth understanding of socio-ecology of PPR in both Eastern and western of D. R. Congo and neighbouring communities is needed in order to address the risk factors associated with cultural, seasonal and socio-economic activities of the involved pastoral areas.

  On strategic-transborder movement emphasize should be on promoting animal movement and trade based on sanitary measures including availability of inspectorate and certification systems for animal ad animal products moved in Central and East Africa. Relevant bodies such as EAC and SADC should be advised too.

- Development of mix PPR-CaP vaccine against the two infections should be pursued for proper control of the spread of these two diseases because they were foun in dual infection in goats and sheep.

- Whole genome sequencing of PPRV isolates from South Kivu should be availed and deposited to the Gene bank for more evolutionary and characterization of PPR viruses in future.

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Appendix I: Competitive Enzyme Linked Immunosorbent Assay (cELISA) using innovative diagnostics kit

(ID vet, France, ID Screen® PPR competition, www.id-vet.com)

1. Cote with PPRV antigen the polystyrene plates (PPR recombinant nucleoprotein)

2. Added to each well, serum sample and monoclonal antibody (mAb) against the N protein of PPRV. Sample dilution factor 1:2

3. Add the secondary antibody labeled with the HRP enzyme. Anti-NP-HRP concentrated conjugate (10)

4. Add substrate to the plate

5. Read the optical density (OD) at 450nm using ELISA DATA Interchange (EDI)

6. Conclusion: Sample Incubation 45 min, Conjugate Incubation 30 min, Three washes and Substrate Incubation 15 min.

7. Test Interpretation S/N < 50% = positive, 50 % < S/N ≤ 60% = doubtful, S/N > 60% =

| Antigen coated on plate | Serum antibodies against PPRV | Substrate |
| Monoclonal antibodies | Secondary antibodies labelled with HRP | |

App
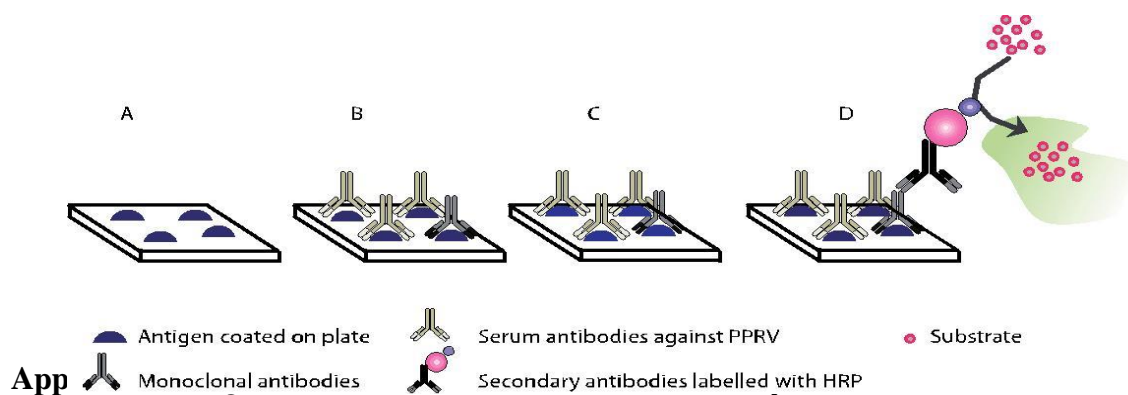
This questionnaire aims to identify people's knowledge and awareness of risk factors that could lead to PPRV transmission at the farm level.

1. **Type of farming system?**

   - Free ranging…………/ - Not free ranking……………

2. **Numbers of livestock keep in your farm?**

Type of animal	Local breed		Improved breed	
	Male	Female	Male	Female
Goats				
Sheep				
Other (specify)				

3. **Geographic coordinate of your herd(location)**

   Altitude (mm)………..……. Longitude...…………..d/m/sec

4. What is the estimation of mortality rate in your herd per year for (for the last 5 past years

   (from 2010-2015)

- Goats?......................percent / Sheep?......................percent

5. What is the estimation of morbidity rate in your herd per year for?

- Goats?......................percent / Sheep?......................percent

6. Have you introduce new animals in your herd? Yes…….No…………….......

7. Which type of grazing system do you apply in your herd?

- Communal grazing…………….Housing………….zero grazing…………..…..

8. Do have access on veterinary services in your village/herd?

   Yes…………………...No……………

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9. Have your animals (goats or sheep) experienced any of the following health problems?

Symptom	Yes	No	Treatment provided
Pneumonia			
Fever / Salivation			
Diarrhea/ Digestive tracts			
Anorexia and			
Depression/nodules			
Nasal and ocular discharges			
difficult respiration			
Necrotic lesions on gum			
Nasal mucosa erosion			
Others (states)…………			

10. Do you import animals in your herds?.................Yes………….No…………....

If yes, what are their origins?

- Animal bought from live animal markets…….……………………………………....

- Animal bought from a far village/region/country location……………………......

- Animal bought from a nearest village/region/country location…………………...

11. Have you ever vaccinated your animal against PPRV? Yes……..No……….….

12. Have you ever vaccinated against others viral diseases? Yes…….No……….…

13. Animals are sharing water sources in your herd? Yes…………No………….…

14. Are you exchanging your animals to other nearest farms for reproductive purposes? Yes…..………………………….……No……………………….….

15. Are goats and sheep rising together in your farm? Yes…………No………..…

16. Characteristics of animal sampled

Parameters	Type of animal	
	Goats	**Sheep**
Sex(M/F)		
Age(month)		
Origin of animal		
Body weight(Kg)		
Height at the withers (Kg)		
chest girth		
body length,		
shoulder width		
pin-bone width		
Breed		
Wind seep of living area		

Appendix III: RNeasy®Plus mini-kit for RNA extraction

For purification of total RNA from animal cells and easy-to-lyse animal tissues using gDNA Eliminator columns.

1. Harvest cells according: Cells grown in suspension (do not use more than 1 x 107 cells): Determine the number of cells. Pellet the appropriate number of cells by centrifuging for 5 min at 300 x g in a centrifuge tube (not supplied). Carefully remove all supernatant by aspiration, and proceed to step 2.
2. Disrupt the cells by adding Buffer RLT Plus. For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add the appropriate volume of Buffer RLT Plus. Vortex or pipet to mix, and proceed to step 3.
3. Homogenize the lysate according.
4. Transfer the homogenized lysate to a gDNA Eliminator spin column placed in a 2 ml collection tube (supplied). Centrifuge for 30 s at ≥8000 x g (≥10,000 rpm). Discard the column, and save the flow-through.
5. Add 1 volume (usually 350 µl or 600 µl) of 70% ethanol to the flow through, and mix well by pipetting. Do not centrifuge.
6. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.*
7. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.* or 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.*
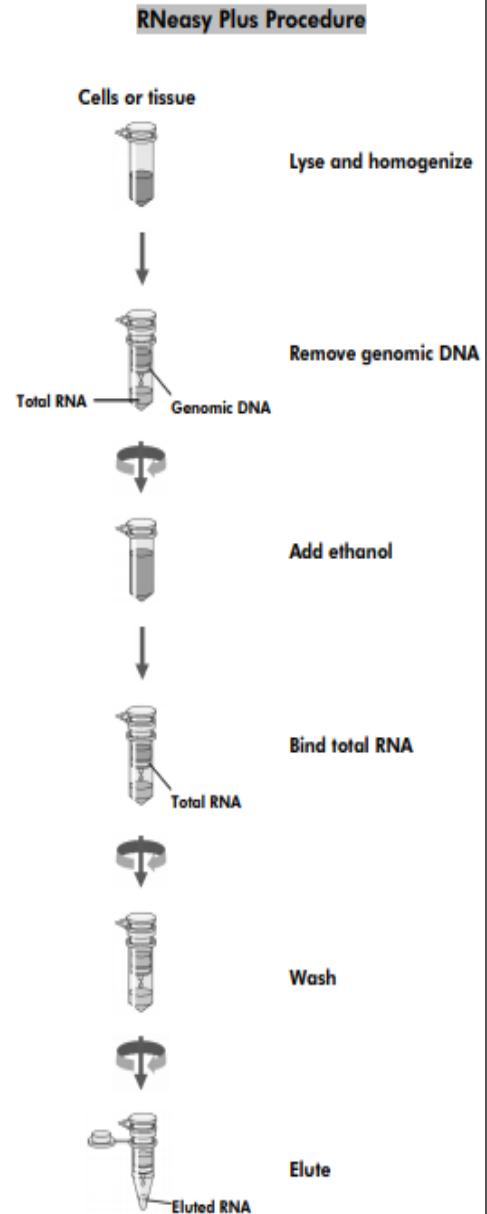
RNeasy Plus Procedure

Cells or tissue

Lyse and homogenize

Remove genomic DNA

Total RNA ← Genomic DNA

Add ethanol

Bind total RNA

Total RNA

Wash

Elute

Eluted RNA

1. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 9.

2. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane.

3. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow through. Centrifuge at full speed for 1 min

4. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.

5. If the expected RNA yield is >30 µg, repeat step 11 using another 30–50 µl of RNase-free water, or using the eluate from step 11 (if high RNA concentration is required). Reuse the collection tube from step 11. If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

Appendix IV: QIAamp viral RNA mini extraction kit

For viral RNA recover from the buffy coat, homogenized plasma, serum cell-free fluids, cell-culture supernatants and whole blood.

1. Add 560 µl of prepared Buffer AVL containing carrier RNA into a 1.5 ml micro centrifuge tube.

2. Add 140 µl plasma, serum, urine, cell-culture supernatant, or cell-free body fluid to the Buffer AVL–carrier RNA in the micro centrifuge tube. Mix by pulse-vortexing for 15 seconds.

3. Incubate at room temperature (15–25°C) for 10 min.

4. Briefly centrifuge the tube to remove drops from the inside of the lid.

5. Add 560 µl of ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.

6. Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.

7. Carefully open the QIAamp Mini column, and repeat step 6.

8. Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW1. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

9. Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min. Continue directly with step 11, or to eliminate any chance of possible Buffer AW2 carryover

10. Recommended: Place the QIAamp Mini column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

11. Place the QIAamp Mini column in a clean 1.5 ml micro centrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µl of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6000 x *g* (8000 rpm) for 1 min. perform step 10, and then continue with step 11.

QIAamp Viral RNA
Mini Spin Procedure

QIAamp Viral RNA
Mini Vacuum Procedure

Sample

Sample

Lyse

Bind

Vacuum

Wash
(Buffer AW1)

Vacuum

Wash
(Buffer AW2)

Vacuum

Elute

Pure viral nucleic acid
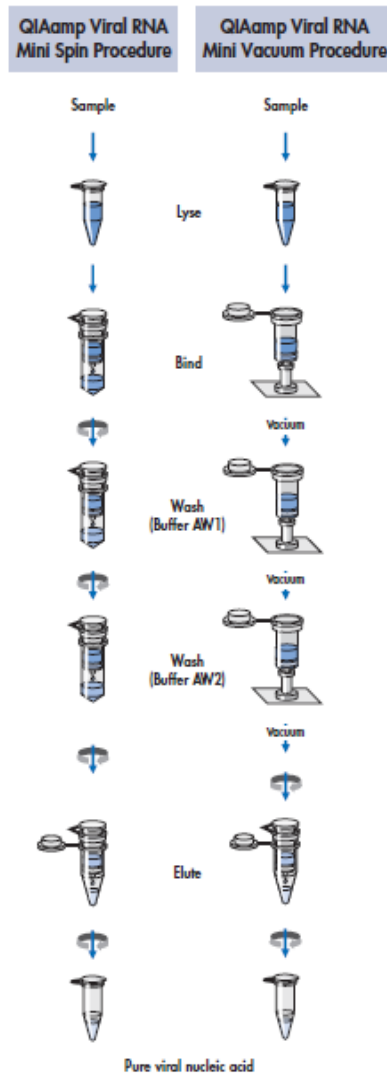
Appendix V: RNA quality and quantity control: bioanalyzer

TapeStation D1000

1. Allow reagents to equilibrate at room temperature for 30 min

2. Vortex mix before use

3. If running ladder, add 3 µL D1000 Sample Buffer to 1 µL D1000 Ladder

4. Add 3 µL D1000 Sample Buffer to 1 µL DNA sample

5. Vortex using IKA vortexer and adaptor at 2000 rpm for 1 min

6. Spin down to position the sample at the bottom of the tube.

Sample Analysis

1. Load samples into the 2200 TapeStation.

2. Select the required samples on the controller software.

3. Click Start and specify a filename with which to save your results.

Appendix VI: Qubit fluorometric RNA quantitation

1. The Qubit® 3.0 Fluorometer is a benchtop fluorometer that can be used for the quantitation of DNA, RNA, microRNA, and protein using the highly sensitive and accurate fluorescence-based Qubit ® quantitation assays.

2. Qubit® RNA HS Assay

3. The Qubit® RNA HS (High Sensitivity) Assay Kits make RNA quantitation easy and accurate. To determine the purity of our samples, we used the Qubit® RNA HS Assay Kit together with the Qubit® dsDNA HS Assay Kit.

4. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit® RNA HS Assay requires 2 standards.

5. Note: Use only thin-wall, clear, 0.5-mL PCR tubes. Acceptable tubes include Qubit® assay tubes (Cat. no. Q32856) or Axygen® PCR-05-C tubes (part no. 10011-830)

6. Label the tube lids.

7. Note: Do not label the side of the tube as this could interfere with the sample read. Label the lid of each standard tube correctly. Calibration of the Qubit® Fluorometer requires the standards to be inserted into the instrument in the right order.

8. Prepare the Qubit® working solution by diluting the Qubit® RNA HS Reagent 1:200 in Qubit® RNA HS Buffer. Use a clean plastic tube each time you prepare Qubit® working solution. Do not mix the working solution in a glass container.

9. Note: The final volume in each tube must be 200 µL. Each standard tube requires 190 µL of Qubit® working solution, and each sample tube requires anywhere from 180–199 µL. Prepare sufficient Qubit® working solution to accommodate all standards and samples. For example, for 8 samples, prepare enough working solution for the samples and 2 standards: ~200 µL per tube in 10 tubes yields 2 mL of working solution (10 µL of Qubit® reagent plus 1990 µL of Qubit® buffer).

10. Add 190 µL of Qubit® working solution to each of the tubes used for standards.

11. Add 10 µL of each Qubit® standard to the appropriate tube, then mix by vortexing 2-3 seconds. Be careful not to create bubbles.

12. Note: Careful pipetting is critical to ensure that exactly 10 µL of each Qubit® standard is added to 190 µL of Qubit® working solution.

13. Add Qubit® working solution to individual assay tubes so that the final volume in each tube after adding sample is 200 µL.

14. Note: Your sample can be anywhere from 1–20 µL. Add a corresponding volume of Qubit® working solution to each assay tube: anywhere from 180–199 µL.

15. Allow all tubes to incubate at room temperature for 2 minutes. Proceed to "Reading standards and samples"; follow the procedure appropriate for your instrument:

Reading standards and samples

1. On the Home screen of the Qubit® 3.0 Fluorometer, press RNA, then select RNA: High Sensitivity as the assay type. The "Read standards" screen is displayed. Press Read Standards to proceed.

2. Note: If you have already performed a calibration for the selected assay, the instrument prompts you to choose between reading new standards and running samples using the previous calibration.

3. Insert the tube containing Standard #1 into the sample chamber, close the lid, then press Read standard. When the reading is complete (~3 seconds), remove Standard #1.

4. Insert the tube containing Standard #2 into the sample chamber, close the lid, then press Read standard. When the reading is complete, remove Standard #2. The instrument displays the results on the Read standard screen. For information on interpreting the calibration results, refer to the Qubit® 3.0 Fluorometer User Guide.

5. Press Run samples.

6. On the assay screen, select the sample volume and units: a. Press the + or − buttons on the wheel to select the sample volume added to the assay tube (from 1–20 μL). b. From the dropdown menu, select the units for the output sample concentration.

7. Insert a sample tube into the sample chamber, close the lid, then press Read tube. When the reading is complete (~3 seconds), remove the sample tube. The instrument displays the results on the assay screen. The top value (in large font) is the concentration of the original sample. The bottom value is the dilution concentration. For information on interpreting the sample results, refer to the Qubit® 3.0 Fluorometer User Guide.

8. Repeat step 6 until all samples have been read.

Qubit® RNA BR Assay

1. The Qubit® RNA BR (Broad-Range) Assay Kits make RNA quantitation easy and accurate. To determine the purity of our samples, we used the Qubit® RNA BR Assay Kit together with the Qubit® dsDNA BR Assay Kit.

2. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit® RNA BR Assay requires 2 standards.

3. Note: Use only thin-wall, clear, 0.5-mL PCR tubes. Acceptable tubes include Qubit® assay tubes (Cat. no. Q32856) or Axygen® PCR-05-C tubes (part no. 10011-830).

4. Label the tube lids.

5. Note: Do not label the side of the tube as this could interfere with the sample read. Label the lid of each standard tube correctly. Calibration of the Qubit® Fluorometer requires the standards to be inserted into the instrument in the right order.

6. Prepare the Qubit® working solution by diluting the Qubit® RNA BR Reagent 1:200 in Qubit® RNA BR Buffer. Use a clean plastic tube each time you prepare Qubit® working solution. Do not mix the working solution in a glass container.

7. Note: The final volume in each tube must be 200 μL. Each standard tube requires 190 μL of Qubit® working solution, and each sample tube requires anywhere from 180–199 μL. Prepare sufficient Qubit® working solution to accommodate all standards and samples. For example, for 8 samples, prepare enough working solution for the samples and 2 standards: ~200 μL per tube in 10 tubes yields 2 mL of working solution (10 μL of Qubit® reagent plus 1990 μL of Qubit® buffer).

8. Add 190 μL of Qubit® working solution to each of the tubes used for standards.

9. Add 10 µL of each Qubit® standard to the appropriate tube, then mix by vortexing 2–3 seconds. Be careful not to create bubbles.

10. Note: Careful pipetting is critical to ensure that exactly 10 µL of each Qubit® standard is added to 190 µL of Qubit® working solution.

11. Add Qubit® working solution to individual assay tubes so that the final volume in each tube after adding sample is 200 µL.

12. Note: Your sample can be anywhere from 1–20 µL. Add a corresponding volume of Qubit® working solution to each assay tube: anywhere from 180–199 µL.

13. Add each sample to the assay tubes containing the correct volume of Qubit® working solution, then mix by vortexing 2–3 seconds. The final volume in each tube should be 200 µL.

14. Allow all tubes to incubate at room temperature for 2 minutes.

15. Proceed to "Reading standards and samples"; follow the procedure described above for Quibit®HS Assay

Appendix VII: cDNA synthesis SuperScript™ II reverse transcriptase

SuperScript™ II Reverse Transcriptase (RT) is an engineered version of MMLV RT with reduced RNase H activity and increased thermal stability. The enzyme is purified to near homogeneity from *E. coli* containing the modified *pol* gene of Moloney Murine Leukemia Virus. The enzyme can be used to synthesize first-strand cDNA at higher temperatures than conventional MMLV RT, providing increased specificity, higher yields of cDNA, and more full-length product. It can generate cDNA up to 12.3 kb.

A 20-μL reaction volume can be used for 1 ng–5 μg of total RNA or 1–500 ng of mRNA.

Add the following components to a nuclease-free microcentrifuge tube:

-Oligo (dT)$_{12-18}$ (500 μg/mL) *or* 50–250 ng random primers *or* 2 pmole gene-specific primer (GSP): 1 μL

-1 ng to 5 μg total RNA *or* 1–500 ng of mRNA: x μL

-1 μL dNTP Mix (10 mM each): 1 μL

-Sterile, distilled water to 12 μL

2. Heat mixture to 65°C for 5 min and quick chill on ice. Collect the contents of the tube by brief centrifugation and add:

- 5X First-Strand Buffer: 4 μL

- 0.1 M DTT: 2 μL

- RNaseOUT™ (40units/μL) (optional)*: 1 μL

*RNaseOUT™ (Cat. No. 10777-019) is required if using <50 ng starting RNA.

3. Mix contents of the tube gently. If you are using oligo (dT)12-18 or GSP, incubate at 42°C for 2 min. If you are using random primers, incubate at 25°C for 2 min.

4. Add 1 μL (200 units) of SuperScript™ II RT and mix by pipetting gently up and down.

If you are using less than 1 ng of RNA, reduce the amount of SuperScript™ II RT to 0.25 µL (50 units) and add sterile, distilled water to a 20 µL final volume. If you are using random primers, incubate tube at 25°C for 10 min.

5. Incubate at 42°C for 50 min.

6. Inactivate the reaction by heating at 70°C for 15 min.

Appendix VIII: RT-PCR amplification of first-strand cDNA synthesis using SuperScript™ II RT

The cDNA obtained in Appendix 8 can now be used as a template for amplification in PCR. However, amplification of some PCR targets (>1 kb) may require the removal of RNA complementary to the cDNA. To remove RNA complementary to the cDNA, add 1 μL (2 units) of *E. coli* RNase H and incubate at 37°C for 20 min.

Use only 10% of the first-strand reaction for PCR. Higher volumes may not increase amplification and may result in decreased amounts of PCR product.

1. Add the following to a PCR tube:

-   10X PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]   :5 μL
-   50 mM MgCl$_2$:1.5 μL
-   10 mM dNTP Mix :1 μL
-   Forward primer (10 μM) :1 μL
-   Reverse primer (10 μM) :1 μL
-   *Taq* DNA polymerase (5 U/μL)   :0.4 μL
-   cDNA from first-strand reaction   :2 μL
-   Autoclaved, distilled water to   :50 μL

2. Mix gently and layer with 1–2 drops (~50 μL) of silicone oil. *(Note: silicone oil is unnecessary in thermal cyclers equipped with a heated lid.).*

3. Heat reaction to 94°C for 2 min to denature.

4. Perform 15 to 40 cycles of PCR. Use the recommended annealing and extension conditions for your *Taq* DNA polymerase.

Appendix IX: QIAquick®Gel extraction kit for PCR products

1. Excise the DNA fragment (PCR amplified fragment) from the agarose gel with a clean, sharp scalpel.

2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg gel ~ 100 µl). The maximum amount of gel per spin column is 400 mg. For >2% agarose gels, add 6 volumes Buffer QG.

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve gel. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The mixture turns yellow.

4. Add 1 gel volume isopropanol to the sample and mix.

5. Place a QIAquick spin column in a provided 2 ml collection tube or into a vacuum manifold. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 µl, load and spin/apply vacuum again.

6. If DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, add 500 µl Buffer QG to the QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.

7. To wash, add 750 µl Buffer PE to QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.

Note: If the DNA will be used for salt-sensitive applications (e.g., sequencing, bluntended ligation), let the column stand 2–5 min after addition of Buffer PE Centrifuge the QIAquick column in the provided 2 ml collection tube for 1 min to remove residual wash buffer.

1. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

2. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.

3. If purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Appendix X: QIAquick PCR purification kit protocol

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene. For example, add 500 μl of Buffer PB to 100 μl PCR samples (not including oil).

2. If pH indicator I has been added to Buffer PB, check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 10 μl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. Place a QIAquick spin column in a provided 2 ml collection tube.

4. To bind DNA, apply the sample to QIAquick column and centrifuge for 30-60s.

5. Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.

6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.

7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.

8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

9. To elute DNA, add 50 μl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1min.

10. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Appendix XI: BigDye™ Terminator v3.1 Cycle Sequencing procedure (Applied Biosystems)

The BigDye ™ Terminator v3.1 Cycle Sequencing Kit provides pre-mixed reagents for Sanger sequencing reactions. The kit reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, on PCR fragments, and on large templates (for example, BAC clones). The kit includes BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer, which is specifically optimized for use with the BigDye™ Ready Reaction mixes. The kit has been formulated to deliver robust performance across a wide variety of DNA sequences while maximizing read lengths. When used in combination with Minor Variant Finder Software, the kit can also be used to detect variants as low as 5% in a sample (see Minor Variant Finder Software User Guide (Pub. No. MAN0014835).

A. Prepare templates

Cycle Sequencing can be performed directly from single– or double–stranded DNA, plasmids, cosmids, BACs or purified PCR products. For high complexity DNA, PCR amplification of the target of interest before cycle sequencing is recommended. PCR templates can also be used to perform reliable cycle sequencing. For optimal results, purify PCR templates before sequencing. In general, any method that removes unincorporated dNTPs and primers should work. Good template quality (free from contaminants) and quantity (Concentration of single-stranded DNA = $A_{260} \times 33$ mg/µL and concentration of double-stranded DNA = $A260 \times 50$ mg/µL).

DNA template	Quantity
PCR product:	
• 100–200 bp	1–3 ng
• 200–500 bp	3–10 ng
• 500–1000 bp	5–20 ng
• 1000–2000 bp	10–40 ng
• >2000 bp	20–50 ng
Single-stranded DNA	25–50 ng
Double-stranded DNA	150–300 ng
Cosmid, BAC	0.5–1.0 µg
Bacterial genomic DNA	2–3 µg

B. Perform cycle sequencing

1. Completely thaw the contents of the BigDye ™ Terminator v3.1 Cycle Sequencing Kit and the primers and store on ice.

2. Vortex the tubes for 2 to 3 seconds, then centrifuge briefly (2 to 3 seconds) with a benchtop microcentrifuge to collect contents at the bottom of the tubes.

3. Add components as indicated:

Component	Standard reaction (20 µL)			Standard reaction (10 µL)		
	Quantity per reaction	Example Forward	Example Reverse	Quantity per reaction	Example Forward	Example Reverse
BigDye™ Terminator 3.1 Ready Reaction Mix	8 µL	8 µL	8 µL	4 µL	4 µL	4 µL
Forward primer (3.2 µM)	3.2 pmol	2 µL	—	3.2 pmol	1 µL	—
Reverse primer (3.2 µM)		—	2 µL		—	1 µL
Deionized water (RNase/DNase-free)	Varies based on template and primer volume	8 µL	8 µL	Varies based on template and primer volume	4 µL	4 µL
Template	See "Template quantity" on page 12	2 µL[1],[2]	2 µL[1],[2]	See "Template quantity" on page 12	1 µL[1], [2]	1 µL[1],[2]
Total volume	20 µL	20 µL	20 µL	10 µL	10 µL	10 µL

[1] e.g. 150-300ng/µL of dsDNA
[2] Concentration of template may affect volume, if template volume differs, adjust the volume of water in the reaction mix.

4.Seal the plate with MicroAmp™ Clear Adhesive Film.

5.Vortex the plate for 2 to 3 seconds, then centrifuge briefly in a swinging bucket centrifuge to collect contents to the bottom of the wells (5 to 10 seconds) at 1,000 x g. Note: Bubbles may be present within the wells, but do not adversely affect the reaction and use BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer to dilute sequencing reactions.

6.Place the tubes or plate(s) in a thermal cycler and set the correct volume: • 20 µL for microcentrifuge tubes or 96-well reaction plates • 10 µL for 384-well reaction plates.

7. Perform cycle sequencing:

Parameter	Stage/step				
	Incubate	25 cycles			Hold
		Denature	Anneal	Extend	
Ramp rate	—	1°C/second			
Temperature	96°C	96°C	50°C	60°C	4°C
Time (mm:ss)	01:00	00:10	00:05	04:00[1]	Hold until ready to purify.

[1] Shorter extension times can be used for short templates.

8.Briefly centrifuge the reactions and proceed to purify the sequencing reactions

C. Purify reactions

The BigDye X Terminator™Purification Kit Protocol (Pub. No. 4374408) takes approximatively 40minutes.

1.Vortex the bottle of BigDye XTerminator™ beads for 8 to 10 seconds before mixing with the SAM solution.

2.Prepare the SAM/BigDye XTerminator™ bead working solution:

Component	Volume per 10 µL reaction	Volume per 20 µL reaction
SAM solution	45 µL	90 µL
BigDye XTerminator™ bead solution	10 µL	20 µL
Total volume	55 µL	110 µL

3. Remove MicroAmp™ Clear Adhesive Film from the sequencing plate.

4. Transfer the indicated volume of bead mix (BigDye XTerminator™ bead solution and SAM solution) to each sample.

5. Seal the plate using MicroAmp™ Clear Adhesive Film.

6. If you are using the Digital Vortex-Genie™ 2, vortex the 96-well plate for 20 minutes at 1,800 rpm. For alternative vortex mixer manufacturers and settings, see the BigDye XTerminator™ Purification Kit Quick Reference Card (Pub. No. 4383427).

7. In a swinging bucket centrifuge, centrifuge the plate at 1,000 x g for 2 minutes.

Note: To store for up to 10 days, seal the plate with MicroAmp™ Clear Adhesive Film, and store at 4°C for capillary electrophoresis (CE) preparation or at –20°C until use. BDX plates can be stored at room temperature for up to 48 hours inclusive of time on the CE instrument.

We can also purify the sequencing reactions with Centri-Sep™ plates or with ethanol/EDTA precipitation

D. Capillary electrophoresis

It is recommended to verify the quality of your current matrix file or spectral calibration before proceeding. To generate a new matrix file or spectral calibration, use the appropriate matrix and/or sequencing standard for your instrument. The electrophoresis can be done on the 310 Genetic Analyzer, on the 3130/3130xl Genetic Analyzer, on the 3500/3500xL Genetic Analyzer or on the 3730/3730xl DNA Analyzer. For this case we used the 310 Genetic Analyzer for electrophoresis as follow:

Item	Module name	Polymer	Capillary length	Mobility files
Dye set E run modules	P4StdSeq (1 mL) E	POP-4™	36-cm	KB_310_POP4_BDTv3_36Std.mob
	P4RapidSeq (1 mL) E	POP-4™	36-cm	KB_310_POP4_BDTv3_36Rapid.mob
	Seq POP6 Rapid (1 mL) E	POP-6™	36cm	KB_310_POP6_BDTv3_36Rapid.mob
	Seq POP6 (1 mL) E	POP-6™	50-cm	KB_310_POP6_BDTv3_50Std.mob

Appendix XII: Peste des petits ruminants virus cultured into vero cells and neutralization.

Cell passage:

Open biological safety hood 15 minutes before using it.

Clean biological safety hood with 70% Ethanol.

Heat cell culture media, PBS 1X and trypsin in a water bath at 37°C for at least 15 minutes before utilization.

Procedure:

1. Remove flask from the incubator at 37°C & 5% CO2.

2. Remove the media inside the flask and discard in a beaker (use 5 ml pipette for the T25 flask and a 10 ml pipette for the T75 flask).

3. Wash cells with PBS 1X (with 5 ml for the T25 flask and 7-10 ml for the T75 flask) to remove the remaining serum that can block trypsin's activity.

4. Remove PBS 1X and discard in the beaker.

5. Add trypsin to the flask (1 ml for the T25 flask and 3 ml for the T75 flask).

6. Incubate the cells with trypsin in the incubator (37°C & 5% CO2) for 5 minutes (VeroSLAM cells take more time to dislodge so incubate for 10 minutes or till cells detach completely from the flask).

7. Remove flask from the incubator.

8. Add complete cell culture media (with fetal bovine serum [FBS]) to the cells to inhibit the trypsin and suspend the cells [with 4 ml of cell culture media for the T25 flask and 5 ml for the T75 flask].

9. Suspend the cells by pipetting up and down for 5 times or till the cells are completely suspended and till no cell clumps are observed.

10. Keep 1 ml of cells in the flask and discard the remaining of the cells in the beaker.

11. Add fresh complete media to the cells in the flask (with 4 ml of media for the T25 flask [final 5 ml] and 9 ml of media for the T75 flask [final 10 ml]. Add Zeocin (1mg/ml) to the "mother flask".

12. Put back the flasks in the incubator ($37^{o}C$ & 5% CO2) and allow the cells to grow for 2-3 days.

Viral sero-neutralization assay:

1. Collect cells as described above up to the step of suspending the cells (step 9).

2. Put cells in a 15 ml Falcon tube.

3. Centrifuge at 2,000 rpm for 5 minutes & discard the supernatant.

4. Suspend the cells in 3-5 ml of fresh complete media (2% FBS).

5. Count live cells diluted 1:10 in Trypan blue using the hemocytometer.

6. Prepare cells according to number of 96-well plates to be used: 2 x 10^6 cells per plate in 5 ml of complete serum in 2 % FBS per plate [so $2x10^4$ cells per well].

7. Keep cells in a Falcon tube till ready to use.

8. Prepare serum dilution as follows:

➤ Put 90 µl of complete media (2% FBS) in each well of the first row (A).

➤ Put 50 µl of complete media (2% FBS) in each well of rows B to H.

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➢ In the first row (A), put 10 µl of serum in 90 µl of complete media (2% FBS) [dilution 1:10].

➢ Prepare quadruplicates for each animal samples.

➢ With multichannel, remove 50 µlof the first row (A) and mix with 50 µl of the second row (B).

➢ Keep the 50 µl in the tips and mix with row C and continue the 1:2 dilutions up to the last row (H).

➢ Discard the remaining 50 µl.

9. Prepare the PPR virus (vaccine strain N75/1) to have 10^2TCID50 per well (so 10^4per plate) in a total of 5 ml of complete media (2% FBS) per plate.

10. Mix the PPR virus with the serum by adding 50 µl of the viral preparation in media to the 50 µl of the different serum dilution in media.

11. Incubate 15 minutes at room temperature.

12. During the 15 min. incubation period, prepare the titration of the virus control ($10^0$to 10^{-4}).

13. Add the cells prepared in step (3) of the "viral sero-neutralization assay procedure" (50 µl of the cell preparation per well).

14. Incubate the plates in the incubator (37^oC & 5% CO2).

15. Read plates at day 3 and day 5-post infection.

Appendix XIII: Total RNA extraction concentration and purity

Sample ID	NA (ng/µl)	A260	A280	260/280	260/230	Factor
1	20,5	0,513	1,077	0,48	0,06	40
2	60,7	1,518	4,195	0,36	0,11	40
3	31,1	0,778	2,035	0,38	0,06	40
4	20,4	0,51	1,39	0,37	0,04	40
5	33	0,824	2,117	0,39	0,08	40
6	11,8	0,295	0,724	0,41	0,02	40
7	27,1	0,677	1,447	0,47	0,05	40
8	17,8	0,445	1,025	0,43	0,04	40
9	19,2	0,481	0,632	0,76	0,09	40
10	46,7	1,167	3,338	0,35	0,09	40
11	17	0,425	0,862	0,49	0,05	40
12	44,4	1,109	2,876	0,39	0,1	40
13	27,9	0,697	1,908	0,37	0,1	40
14	26,5	0,661	1,771	0,37	0,06	40
15	27,3	0,683	1,738	0,39	0,12	40
16	40	0,999	2,489	0,4	0,24	40
17	37,7	0,943	2,203	0,43	0,1	40
18	27,5	0,687	1,684	0,41	0,06	40
19	37,4	0,934	2,696	0,35	0,07	40
20	36,7	0,918	2,712	0,34	0,07	40
21	18	0,45	1,095	0,41	0,04	40
22	15,8	0,395	1,053	0,38	0,1	40
23	50,7	1,268	3,564	0,36	0,1	40
24	14,9	0,373	0,74	0,5	0,03	40
25	12	0,301	1,023	0,29	0,04	40
26	35,1	0,879	1,746	0,5	0,28	40
27	15,7	0,392	1,067	0,37	0,03	40
28	9,2	0,23	0,699	0,33	0,12	40
29	23,4	0,584	1,431	0,41	0,05	40
30	10,2	0,255	0,754	0,34	0,04	40

31	26,5	0,663	1,955	0,34	0,06	40
32	13,3	0,332	1,038	0,32	0,09	40
33	55,6	1,389	4,456	0,31	0,17	40
34	15,8	0,394	1,386	0,28	0,03	40
35	40,3	1,008	3,125	0,32	0,18	40
36	54,5	1,361	4,138	0,33	0,1	40
37	39,8	0,994	2,917	0,34	0,08	40
38	26,8	0,669	1,972	0,34	0,05	40
39	18	0,449	1,275	0,35	0,04	40
40	90	2,25	0,691	3,26	1,81	40
41	64,2	1,605	0,584	2,75	0,62	40
42	10,8	0,271	0,119	2,27	0,46	40
43	159,3	3,982	1,712	2,33	1,52	40
44	**634,8**	**15,87**	**7,388**	**2,15**	**0,47**	**40**
45	60,1	1,504	0,475	3,17	0,7	40
46	205,4	5,134	2,462	2,09	0,77	40
47	38,2	0,956	0,305	3,14	0,16	40
48	25,7	0,643	0,21	3,06	0,85	40
49	11,9	0,297	0,146	2,03	0,41	40
50	25,9	0,646	0,217	2,98	0,08	40
51	15,6	0,391	0,201	1,95	0,11	40
52	31,7	0,792	0,367	2,16	0,48	40
53	17,6	0,441	0,168	2,62	0,11	40
54	50,1	1,252	0,612	2,05	1,13	40
55	79	1,975	0,627	3,15	0,99	40
56	50,8	1,27	0,405	3,14	1,01	40
57	205	5,137	2,467	2,08	0,76	40
58	40.2	1.005	1.394	0.72	0.28	40
59	57.1	1.427	1.876	0.76	0.29	40
60	39.5	0.988	0.78	1.27	0.43	40
61	55.3	1.383	2.902	0.48	0.1	40
62	61.8	1.545	3.048	0.51	0.18	40
63	35.1	0.878	1.424	0.62	0.14	40

64	30.6	0.766	1.387	0.55	0.06	40
65	91.6	2.291	6.366	0.36	0.23	40
66	70.1	1.753	2.437	0.72	0.3	40
67	88.1	2.202	6.206	0.35	0.2	40
68	69	1.725	2.944	0.59	0.2	40
69	71.6	1.789	5.153	0.35	0.19	40
70	241.5	6.039	14.177	0.43	0.32	40
71	91.4	2.286	5.122	0.45	0.22	40
72	159.7	3.993	9.696	0.41	0.3	40
73	53.1	1.327	2.737	0.49	0.27	40
74	180	4.499	10.532	0.43	0.37	40
75	231.5	5.787	13.787	0.42	0.34	40
76	211.9	5.297	12.583	0.42	0.37	40
77	169.4	4.234	8.851	0.48	0.36	40
78	154.1	3.852	10.042	0.38	0.27	40
79	241.1	6.027	14.126	0.43	0.33	40
80	154.8	3.869	10.194	0.38	0.23	40
81	151.7	3.794	11.015	0.34	0.29	40
82	125.5	3.138	7.432	0.42	0.36	40
83	294.7	7.369	16.67	0.44	0.42	40
84	275.9	6.897	16.227	0.43	0.37	40
85	166.3	4.158	11.563	0.36	0.23	40
86	165.3	4.131	11.082	0.37	0.33	40
87	237.8	5.945	15.091	0.39	0.35	40
88	262.9	6.571	15.89	0.41	0.36	40
89	184.3	4.608	12.205	0.38	0.33	40
90	166.5	4.162	11.5	0.36	0.26	40
91	193.1	4.828	13.252	0.36	0.28	40
92	210.7	5.268	13.641	0.39	0.29	40
93	494.3	12.357	16.465	0.75	0.77	40
94	270.4	6.76	16.118	0.42	0.37	40
95	232.3	5.807	14.325	0.41	0.34	40
96	173.6	4.339	11.288	0.38	0.32	40

97	295.5	7.389	17.133	0.43	0.39	40
98	385.3	9.632	17.778	0.54	1.05	40
99	453.9	11.348	17.268	0.66	1.73	40
100	116.1	2.902	8.099	0.36	0.18	40
101	127.6	3.19	8.789	0.36	0.18	40
102	150.6	3.765	9.797	0.38	0.25	40
103	64.4	1.61	0.797	2.02	0.27	40
104	105.4	2.635	1.278	2.06	1.38	40
105	17.9	0.448	0.297	1.51	0.17	40
106	18.8	0.471	0.318	1.48	0.29	40
107	402.4	10.06	4.94	2.04	2.16	40
108	37.9	0.948	0.495	1.92	1.93	40
109	10.4	0.259	0.122	2.12	0.84	40
110	36.8	0.737	0.411	1.79	0.12	50
111	30.6	0.764	0.419	1.82	0.1	40
112	20.3	0.508	0.276	1.84	0.11	40
113	20.1	0.503	0.269	1.87	0.1	40
114	13	0.324	0.153	2.13	0.58	40
115	22.9	0.572	0.704	0.81	0.12	40
116	42.9	1.072	0.521	2.06	0.28	40
117	24.6	0.616	0.266	2.32	0.32	40
118	84.7	2.117	1.049	2.02	0.42	40
119	30.9	0.773	0.396	1.95	0.79	40
120	11.4	0.284	0.135	2.1	0.28	40
121	29.5	0.737	0.342	2.16	0.15	40
122	16.5	0.413	0.173	2.38	0.04	40
123	12.5	0.313	0.124	2.52	0.06	40
124	12.6	0.316	0.142	2.23	0.14	40
125	48.2	1.204	0.623	1.93	0.99	40
126	22.3	0.559	0.246	2.27	0.08	40
127	27.3	0.682	0.343	1.99	0.07	40
128	**10.2**	**0.256**	**0.114**	**2.24**	**0.1**	**40**
129	25	0.626	0.32	1.95	0.54	40

130	10.4	0.259	0.109	2.38	0.1	40
131	13	0.324	0.161	2.01	0.22	40
132	17.7	0.443	0.226	1.96	0.18	40
133	13	0.324	0.17	1.91	0.24	40
134	31.3	0.782	0.418	1.87	1.17	40
135	25.5	0.637	0.335	1.9	0.14	40
136	17.4	0.436	0.22	1.99	0.05	40
137	17.4	0.435	0.218	2	0.04	40
138	110	2.718	1.268	2.15	1.13	40
139	12.9	0.323	0.157	2.05	0.12	40
140	91.5	2.287	0.991	2.31	0.14	40
141	108.6	2.715	1.266	2.14	1.12	40
142	98.1	2.452	1.047	2.34	0.31	40
143	76.5	1.914	0.798	2.4	1.36	40
144	39.9	0.998	0.483	2.07	1.49	40
145	124.1	3.102	1.416	2.19	0.46	40
146	143.4	3.585	1.45	2.47	0.93	40
147	60.2	1.506	0.534	2.82	0.12	40
148	11.1	0.278	0.096	2.88	0.37	40
149	15.6	0.39	0.145	2.7	0.08	40
150	13.5	0.339	0.122	2.78	0.07	40

NA: Nucleic Acid concentration

Appendix XIV: DNeasy® blood&tissue extraction protocol (QIAGEN)

This protocol can be used for purification of total DNA from animal blood animal tissue rodent tails ear punches cultured cells fixed tissue bacteria insects.
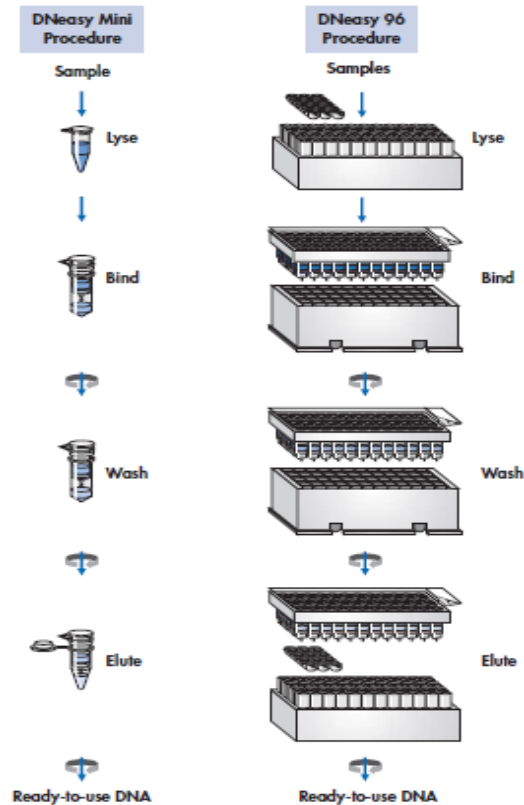
1.   For blood with nonnucleated erythrocytes, follow step 1a; for blood with nucleated erythrocytes, follow step 1b; for cultured cells, follow step 1c.

1a. Nonnucleated: Pipet 20 μl proteinase K into a 1.5 ml or 2 ml microcentrifuge tube (not provided). Add 50–100 μl anticoagulated blood. Adjust the volume to 220 μl with PBS. Continue with step 2.

1b. Nucleated: Pipet 20 μl proteinase K into a 1.5 ml or 2 ml microcentrifuge tube (not provided). Add 5–10 μl anticoagulated blood. Adjust the volume to 220 μl with PBS. Continue with step 2.

1c. Cultured cells: Centrifuge the appropriate number of cells (maximum 5 x 106) for5 min at 300 x g. Resuspend the pellet in 200 μl PBS. Add 20 μl proteinase K. Continue with step 2.

2. Add 200 μl Buffer AL (without added ethanol). Mix thoroughly by vortexing, and incubate at 56°C for 10 min.

3. Add 200 μl ethanol (96–100%) to the sample, and mix thoroughly by vortexing

4. Pipet the mixture from step 3 into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at _6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.

5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 μl Buffer AW1, and centrifuge for 1 min at _6000 x g (8000 rpm). Discard flow-through and collection tube,

6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 μl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube

7. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 μl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at _6000 x g(8000 rpm) to elute.

8. Recommended: For maximum DNA yield, repeat elution once as described in step7

Appendix XV: Salt DNA extacion method

During the extraction of deoxyribonucleic acid, or DNA, salt compounds such as sodium acetate and ammonium acetate are typically added to aid in the removal of DNA-associated proteins. Another type of salt compound called sodium chloride, or NaCl, helps in solidifying and making DNA visible. When mixed in an alcohol solution, the sodium component of NaCl provides a protective barrier around the negatively-charged DNA phosphate ends, enabling them to move closer to be extracted out of the solution.

For fresh or frozen blood samples we used this method following the procedure bellow:

1. Allow to thaw at room temperature).

2. Mix 10 ml of blood with 40 ml of lysis buffer in a 50 ml tube and place on ice for 10minutes

3. Centrifuge at 3, 500 rpm for 10 minutes at $4^{o}C$

4. Discard the supernatant, add 9 ml of solution B and vortex to re-suspend the white pellet

5. Repeat steps to 2 & 3 and discard the supernatant

6. Add 4.5 ml of solution B and re-suspend pellet by vortexing vigorously

7. Add 0.3 ml of proteinase-K/ 10% SDS solution

8. Incubate overnight at $55\,^{o}C$

9. Add 1.4 ml of saturated NaCl and vortex vigorously for 15 seconds

10. Centrifuge at 3000 rpm for 10 minutes at $4^{o}c$

11. Transfer supernatant to a new labeled tube and measure volume. Repeat step 9 if supernatant is not clear

12. Add 2 times (volume) of cold absolute ethanol and mix by gentle inversion (DNA can be seen at this stage as a white precipitate)

13. Remove DNA with a pipette and transfer to a new labeled 50 ml tube

14. Wash DNA with 1 ml of 80% ethanol

15. Air-dry the DNA (Be careful not to over-dry it as it will make re-suspension difficult)

16. Re-suspend the DNA in Tris-EDTA buffer

Reagents:

a) Lysis Buffer

Sucrose   109.536g

1M Tris-HCL (Ph 7.5)   10ml

1M MgCl2   5ml

Triton-X 100 (Add last)   10ml

Top up with autoclaved water to make 1L

c) Solution B

0.5M NaCl   15ml

0.5M EDTA (pH 8.0) 5ml

   Top up with autoclaved water to

      make 1L

b) Proteinase-K/10%SDS

10% SDS   0.5ml

Proteinase-k stock (8mg/ml)   0.5ml

d) Saturated NaCl

NaCl   379.6g

Heat to dissolve in autoclaved water

to make 1L

Appendix XVI: Genomic DNA extraction concentration and purity

Sample ID	NA (ng/µl)	A260	A280	260/280	260/230	Factor
1	148.6	2.972	1.61	1.85	1.92	50
2	37.7	0.754	0.424	1.78	1.01	50
3	44.4	0.888	0.487	1.82	1.61	50
4	94	1.881	1.005	1.87	1.7	50
5	55.2	1.103	0.62	1.78	1.2	50
6	102.3	2.046	1.11	1.84	1.74	50
7	65.1	1.302	0.716	1.82	1.43	50
8	9.7	0.195	0.11	1.77	0.39	50
9	**7.5**	**0.151**	**0.06**	**2.52**	**0.56**	**50**
10	74.8	1.496	0.816	1.83	1.75	50
11	81.4	1.627	0.882	1.84	1.75	50
12	15.8	0.316	0.16	1.97	0.58	50
13	51.9	1.038	0.569	1.83	1.46	50
14	119.7	2.393	1.309	1.83	1.89	50
15	88.6	1.773	0.984	1.8	1.47	50
16	39.7	0.794	0.423	1.88	1.15	50
17	13.6	0.271	0.147	1.85	1.1	50
18	12.7	0.254	0.128	1.98	0.97	50
19	89.2	1.785	0.952	1.88	1.82	50
20	69.2	1.384	0.758	1.83	1.74	50
21	65.7	1.315	0.71	1.85	0.84	50
22	67.2	1.343	0.726	1.85	1.66	50
23	111.4	2.229	1.204	1.85	2	50
24	20	0.4	0.23	1.74	0.57	50
25	63.3	1.267	0.695	1.82	1.58	50
26	49.1	0.981	3.074	0.32	0.31	50
27	61.4	1.227	3.78	0.32	0.31	50
28	51.9	1.038	0.739	1.4	0.59	50
29	61.2	1.223	0.725	1.69	0.77	50
30	620.3	12.407	7.217	1.72	1.24	50
31	1234.5	24.69	13.307	1.86	2.19	50
32	1091.7	21.833	12.053	1.81	1.8	50
33	811.5	16.229	9.099	1.78	1.47	50
34	569.8	11.395	6.289	1.81	1.7	50
35	61.5	1.228	3.79	0.33	0.31	50
36	180.2	3.604	4.372	0.82	0.82	50
37	962	19.239	10.575	1.82	1.74	50

38	978.3	19.566	10.766	1.82	1.85	50
39	14.7	0.294	0.177	1.67	0.33	50
40	297.3	5.947	3.226	1.84	1.96	50
41	1037.4	20.748	11.392	1.82	1.83	50
42	136.8	2.736	1.483	1.83	1.92	50
43	1864.9	37.297	20.731	1.8	1.74	50
44	1504	30.079	16.581	1.81	1.93	50
45	512	10.239	5.675	1.8	1.68	50
46	22.8	0.456	0.248	1.84	2.06	50
47	135.9	2.719	1.477	1.84	1.53	50
48	71.9	1.437	0.792	1.81	1.49	50
49	106.5	2.129	1.172	1.82	1.44	50
50	16.8	0.336	0.171	1.96	1.03	50
51	136.9	2.737	1.484	1.84	1.93	50
52	76.9	1.537	0.845	1.82	1.57	50
53	79.1	1.583	0.867	1.83	0.27	50
54	129.17	2.583	1.348	1.92	0.95	50
55	1142.64	22.853	12.178	1.88	1.94	50
56	136.02	2.72	1.944	1.4	1.88	50
57	269.14	5.383	3.187	1.69	1.35	50
58	336.6	6.733	3.67	1.85	1.42	50
59	115.5	2.31	1.202	1.92	2.16	50
60	175.88	3.518	2.383	1.48	7.15	50
61	126.22	2.524	1.328	1.9	1.02	50
62	243.97	4.879	2.913	1.67	3.91	50
63	37.06	0.741	0.405	1.83	0.77	50
64	753.46	15.069	8.573	1.76	1.28	50
65	62.82	1.256	5.241	0.24	0.78	50
66	17.36	0.347	0.196	1.77	2.15	50
67	42.72	0.854	0.44	1.94	0.92	50
68	37.1	0.742	0.363	2.04	4.63	50
69	51.36	1.027	0.52	1.98	0.75	50
70	115.34	2.307	1.564	1.47	1.87	50
71	336.59	6.732	3.66	1.84	1.41	50
72	57.31	1.146	0.602	1.9	0.57	50
73	225.4	4.508	2.365	1.91	1.7	50
74	232.4	4.648	2.472	1.88	1.63	50
75	249.62	4.992	2.641	1.89	1.57	50
76	72.59	1.452	0.747	1.94	0.88	50

77	161.53	3.231	1.685	1.92	0.94	50
78	202.8	4.056	2.523	1.61	4.1	50
79	155.03	3.101	1.623	1.91	1.52	50
80	47.91	0.958	0.517	1.85	1.73	50
81	91.91	1.838	0.993	1.85	1.19	50
82	226.68	4.534	2.393	1.89	1.55	50
83	125.1	2.502	1.278	1.96	0.96	50
84	68.77	1.375	0.712	1.93	3.61	50
85	222.57	4.451	2.338	1.9	1.18	50
86	580.4	11.608	6.105	1.9	1.26	50
87	3037.22	60.744	33.32	1.82	1.96	50
88	276.64	5.533	3.326	1.66	3.6	50
89	133.87	2.677	1.398	1.92	1.76	50
90	188	3.76	2.414	1.56	2.17	50
91	172.19	3.444	2.232	1.54	2.96	50
92	202.8	4.056	2.523	1.61	4.1	50
93	343.8	6.876	3.656	1.88	2.12	50
94	1599.42	31.988	17.639	1.81	1.47	50
95	24.96	0.499	0.231	2.16	0.64	50
96	204.04	4.081	2.418	1.69	3.11	50
97	412.79	8.256	4.477	1.84	2.04	50
98	305.65	6.113	3.245	1.88	2.19	50
99	322.18	6.444	3.943	1.63	1.61	50
100	11.92	0.238	0.102	2.34	3.08	50
101	124.88	2.498	8.448	0.3	0.51	50
102	222.83	4.457	2.626	1.7	2.57	50
103	839.79	16.796	9.024	1.86	1.53	50
104	40.42	0.808	-0.347	-2.33	-0.27	50
105	277.58	5.552	3.341	1.66	2.33	50
106	879.59	17.592	9.338	1.88	1.67	50
107	930.72	18.614	9.839	1.89	1.68	50
108	**1892.38**	**37.848**	**20.744**	**1.82**	**1.66**	**50**
109	282.7	5.654	3.236	1.75	2.09	50
110	313.49	6.27	3.214	1.95	1.82	50
111	202.42	4.048	10.213	0.4	5.79	50
112	227.56	4.551	2.413	1.89	2.23	50
113	185.62	3.712	2.013	1.84	1.16	50
114	931.73	18.635	9.827	1.9	1.69	50
115	100.37	2.007	1.036	1.94	2.32	50

116	221.85	4.437	2.631	1.69	2.21	50
117	304.22	6.084	3.63	1.68	2.31	50
118	208.3	4.166	2.247	1.85	1.51	50
119	1609.1	32.182	17.286	1.86	2.02	50
120	90.7	1.814	0.997	1.82	0.69	50
121	55.1	1.102	0.596	1.85	0.73	50
122	104.8	2.097	1.152	1.82	1.28	50
123	1263.7	25.274	13.888	1.82	1.66	50
124	58.6	1.172	0.639	1.83	0.65	50
125	49	0.98	0.531	1.84	0.39	50
126	586.1	11.723	6.596	1.78	1.92	50
127	347	6.94	3.76	1.85	1.73	50
128	578.5	11.57	6.323	1.83	1.99	50
129	1616.4	32.328	18.081	1.79	1.47	50
130	1367.5	27.349	15.391	1.78	1.36	50
131	285.2	5.705	3.076	1.85	1.87	50
132	1888.9	37.778	22.435	1.68	1.01	50
133	881.5	17.629	9.654	1.83	1.89	50
134	777.2	15.543	8.408	1.85	1.97	50
135	471.4	9.428	5.308	1.78	1.36	50
136	679.4	13.587	7.712	1.76	1.29	50
137	120.5	2.411	1.311	1.84	1.35	50
138	94.4	1.889	1.052	1.8	0.57	50
139	147.6	2.952	1.601	1.84	0.97	50
140	1043.5	20.871	11.503	1.81	1.68	50
141	64	1.28	0.7	1.84	2.42	50
142	40.9	0.819	0.44	1.85	2.63	50
143	44.4	0.888	0.47	1.91	2.99	50
144	10	0.201	0.09	2.18	4.53	50
145	43.7	0.873	0.47	1.86	3.13	50
146	44.5	0.89	0.48	1.86	3.05	50
147	32.5	0.65	0.35	1.85	3.31	50
148	28.4	0.568	0.32	1.8	3.45	50
149	112.2	2.243	1.21	1.86	2.68	50
150	24.1	0.482	0.26	1.84	2.78	50

NA: Nucleic Acid concentration

Appendix XVII: Questionnaire based survey for PPR socio-economic impact.

Questionnaire ID:………………………………………………………………………...

Location……………………County……………….Village……………………

Geographic coordinates:……………………………………………………………….

Sex of animals keeper………….Female………………….Male………………..

Number of PPR outbreaks reported in the last 5years (from 2011-2015)……………......

1. What the total number of animals possessed in your farm during the last past years?

Years	Goats kids		Sheep (Lamb)		Old Goat		Old sheep	
	F	M	F	M	F	M	F	M
2011								
2012								
2013								
2014								
2015								
Total								

Young (kids):<12months , Old animals : >12months

2. How many animals died in your farm from suspected PPR during the five past years?

Years	Goats kids		Sheep (Lamb)		Old Goat		Old sheep	
	F	M	F	M	F	M	F	M
2011								
2012								
2013								
2014								
2015								
Total								

Young (kids):<12months , Old animals : >12months

3. List the names of others animals (species) in your farm apart from goats and sheep:

A. ………………………………………………………………

B. ………………………………………………………………

C. ………………………………………………………………

D. ………………………………………………………………

4. What is the average selling price of both healthy and infected animals?

Selling price (FC)	Goats kids	Sheep (Lamb)	Old Goat	Old sheep
Healthy animals				
Sick animals				
Total				

Young (kids):<12months , Old animals : >12months , FC (Congolese franc).

5. How many animals survived in your farm after PPR outbreaks during the five past years?

Years	Goats kids		Sheep (Lamb)		Old Goat		Old sheep	
	F	M	F	M	F	M	F	M
2011								
2012								
2013								
2014								
2015								
Total								

Young (kids):<12months , Old animals : >12months

6. From the survived animals how may had a low selling price compare to the normal selling price?

Years	Goats kids		Sheep (Lamb)		Old Goat		Old sheep	
	F	M	F	M	F	M	F	M
2011								
2012								
2013								
2014								
2015								
Total								

Young (kids):<12months , Old animals : >12months

7. What is the number of animals that have lost weight due to PPR in the last past 5years

Years	Goats kids (kg)		Lamb (kg)		Old Goat (Kg)		Old sheep (Kg)	
	F	M	F	M	F	M	F	M
2011								
2012								
2013								
2014								
2015								
Total								

Young (kids):<12months , Old animals : >12months

8. What was the estimated average losses of weight per animal expressed in (kg) for animals infected and survived /recovered with PPR?

Years	Goats kids (kg)		Lamb (kg)		Old Goat (Kg)		Old sheep (Kg)	
	F	M	F	M	F	M	F	M
2011								
2012								
2013								
2014								
2015								
Total								

Young (kids):<12months , Old animals : >12months

9. What was the average selling price of kg to market during the last past years?

Years	Goats kids (kg)		Lamb (kg)		Old Goat (Kg)		Old sheep (Kg)	
	F	M	F	M	F	M	F	M
2011								
2012								
2013								
2014								
2015								
Total								

Young (kids):<12months , Old animals : >12months

10.   What was the number of females having aborted during the past five years ?

Years	Goats kids (kg)		Lamb (kg)		Old Goat (Kg)		Old sheep (Kg)	
	F	M	F	M	F	M	F	M
2011								
2012								
2013								
2014								
2015								
Total								

Young (kids):<12months , Old animals : >12months

List of publications

1. **Bwihangane A Birindwa**, Gitao C George, Bebora C Lilly, Getinet M Tarekegn, Nicholas Svitek, Bacigale Samy. Mitochondrial DNA variation of indigenous goat populations from *Peste-des-petits ruminants* outbreak in South Kivu, Democratic Republic of the Congo. Livestock Research for Rural Development 30 (1) 2018

2. **Birindwa BA,** George GC, Ntagereka BP, Christopher O, Lilly BC. Mixed infection of peste-des-petits ruminants and Capripox in goats in South Kivu, Democratic Republic of Congo. Journal of Advanced Veterinary and Animal Research. 2017; 4(4):348-355.

3. **Bwihangane BA,** Gitao CG, Bebora LC and Nicholas S, 2016. Current Knowledge on Peste des petits ruminants: A comprehensive review on clinical signs, diagnostic test and vaccination. Res. Opin. Anim. Vet. Sci., 7(3): 58-66

4. **Bwihangane, B.A,** Misinzo, G., Sviteck, N., Bebora, L.C 4 & George, C.G. 2017: Sero-epidemiology of Peste des petits ruminants infection and the associated risk factors in South Kivu, DR. Congo. RUFORUM Working Document Series (ISSN 1607- 9345) No. 14 (2): 737 - 746. Available from *http://repository.ruforum.org*

5. **Bwihangane AB,** Gitao CG and Bebora CL, 2017. Economic losses associated with peste des petits ruminants in South Kivu Province of Democratic Republic of Congo. Res. Opin. Anim. Vet. Sci., 7(12): 77-84.