PARTIAL MOLECULAR CHARACTERIZATION OF PESTE DES PETITS RUMINANTS VIRUS IN GOATS OF NGORONGORO DISTRICT, TANZANIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH MOLECULAR BIOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

Livestock sector in the South African Development Community (SADC) accounts for 20 to 40% of agricultural gross domestic products holding high social values of the rural communities in the region of which 60% of the population depend on livestock. However, the sector is hampered by a multitude of economically important diseases including peste des petits ruminants (PPR). The disease is an acute highly contagious viral disease of domestic and wild ruminants caused by PPR virus (PPRV). It was first reported in West Africa in 1940s and confined there for decades. Afterwards, PPR spread to several other parts of the world including Asia and Europe. In Tanzania, PPR was officially reported in 2008 in the northern zone and in 2011 it was reported in the southern zone. The present study was conducted in Ngorongoro District of northern Tanzania to confirm the continued presence of PPRV and to partially characterize the PPRV circulating in northern Tanzania. A total of 28 goat samples (tissues, whole blood and swabs) were collected and analyzed. PPRV was detected in seven goats (25%) in all sample types (blood, tissue and swabs) using reverse transcription polymerase chain reaction (RT-PCR). PPRV nucleotide (N) gene nucleotide sequence analysis clustered PPRV circulating in northern Tanzania into linage III with the percentage nucleotide identity of 95% to isolates from Ethiopia. Findings from this study indicates that swabs and whole blood are useful clinical samples for screening of PPRV especially in areas where animals may not exhibit clinical signs and carcasses are difficult to find. The continued circulation of PPRV in Ngorongoro District warrants further studies in order to determine whether wild animals serve as reservoirs of PPR.

DECLARATION

I, Emmanuel Sadikiel Macha do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted to any other institution.

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DEDICATION

I dedicate this work to my lovely wife Mrs. Emmanuela, my daughter Hoyce Emmanuel and son Hans Emmanuel for their unconditional love that brought happiness, courage and confidence throughout this study.

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	degree Celsius
x *g*	centrifuge rotor speed
bp	base pair
BLAST	Basic Local Alignment Search Tool
cELISA	competitive enzyme-linked immunosorbent assay
DNA	deoxyribonucleic acid
EMPRES	Emergency Prevention System for Transboundary Animal and Plant Pests and Diseases
F	fusion protein
FAO	Food and Agriculture Organization of the United Nations
H	haemagglutinin protein
L	polymerase protein
M	matrix protein
MoLFD	Ministry of livestock and fisheries development
mg	milligram
min	minutes
ml	millilitre
mRNA	messenger ribonucleic acid
N	nucleoprotein
NBS	National Bureau of Statistics
NCBI	National Center for Biotechnology Information
NP3/NP4	nucleoprotein primer set
OIE	Office International des Epizooties
P	phosphoprotein

PCR	polymerase chain reaction
PPR	peste des petits ruminants
PPRV	peste des petits ruminants virus
PPRV F1/F2	fusion protein primer set
RdRp	RNA dependent RNA polymerase
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SADC	Southern African Development Community
TADs	transboundary animal diseases
TAE	Tris acetate-ethyldiamine tetraacetic acid buffer
WHO	World Health Organization of the United Nations
µl	microlitre

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Peste des petits ruminants (PPR) is an acute highly contagious viral disease of domestic and wild ruminants caused by PPR virus (PPRV), a *Morbillivirus* belonging to the family *Paramyxoviridae* (Kwiatek *et al.*, 2010). Peste des petits ruminanats virus is antigenically and biologically related to rinderpest virus and clinically mimics rinderpest in goats and sheep (Luka *et al.*, 2011). Genetically, PPRV isolates are clustered into four distinct lineages (I-IV) on the basis of partial sequence analysis of the nucleoprotein (N) and or fusion (F) protein genes (Dhar *et al.*, 2002). Lineage I and II is found in West Africa, whilst linage III is found in East Africa as well as in Sudan, Ethiopia, Eritrea and some few areas of Asia in Arabian Peninsula and Middle East (Ashley *et al.*, 2010). Lineage IV is the major strain affecting many areas of Central Africa, North Africa, and Asia (Ashley *et al.*, 2010). However, lineage IV has been also reported to be responsible for the 1996 PPR outbreak in Turkey and Uganda (Luka *et al.*, 2011). Peste des petits ruminants manifests clinically with fever, diarrhoea, oculo-nasal discharges, erosive stomatitis and formation of crusting scabs along the lips. In late stages of PPR, development of pneumonias may occur resulting in high mortality rates (EMPRES, 2009). Morbidity and mortality rates vary considerably and may be as high as 90-100% depending on the susceptibility of small ruminants population in the area, breed, type of husbandry and age (Ezeokoli *et al.*, 1986). Peste des petits ruminants is transmitted by direct contact involving secretions or excretions from infected animals to healthy animals in close proximity especially when grazing in communal areas.

Several methods and techniques that either detects the virus or antibodies are available for the diagnosis of PPR. These techniques include agar gel immunodiffusion (AGID), counter immunoelectrophoresis (CIEP), immunocapture and sandwich enzyme-linked

immunosorbent assay (ELISA), culture and virus isolation. Other methods are virus neutralization and competitive ELISA (OIE, 2008). However, recently, molecular biology tools have made it possible to diagnose PPR rapidly and with great sensitivity compared to earlier tests. One of the most sensitive and rapid molecular biology tools is the amplification of the viral nucleic acid by reverse transcription polymerase chain reaction (RT-PCR).

Peste des petits ruminants was first reported in West Africa in 1942 (Gargadennec and Lalanne, 1942) and from that time it spread to other parts of the world. However, it is endemic in sheep and goat populations in Asia, China, Middle East, Eastern parts of Europe, West, Central and East Africa (Ashley *et al.*, 2010; Ezeibe *et al.*, 2008). In East Africa, serological detection of PPR was evidenced in Kenya and Uganda (Wamwayi *et al.*, 1995). Later on, the clinical disease was confirmed in the two countries in 2007 (EMPRES, 2009). The disease was first reported in Tanzania in 2008 when it was confined to the northern zone in districts bordering Kenya (Kivaria *et al.*, 2009). However, a retrospective study using archived samples collected in northern Tanzania demonstrated the presence of PPR antibodies in Ngorongoro District as far as 2004 (Karimuribo *et al.*, 2011). Later on, the disease spread to other parts of the country and PPR has recently been confirmed in southern Tanzania (Muse *et al.*, 2012). Other results have detected antibodies against PPRV from cattle sampled in 2011 in northern Tanzania within the Loliondo Game Controlled Area (Lembo *et al.*, 2013).

Vaccination has remained to be the only feasible PPR control option because of the inability to afford the zoo-sanitary control measures like imposing quarantine to control animal movement especially in pastoral communities (Diallo, 2004). In an effort to control PPR, vaccination was conducted in the northern zone of Tanzania since 2009 by the

Ministry of Livestock and Fisheries Development (MoLFD) in collaboration with the Local Government Authorities and private sector. However, the effectiveness of PPR vaccination depends on the level of vaccination coverage. The low vaccination coverage (32.0%) in affected areas in Ngorongoro reported in 2010 is far below the minimum recommended coverage of 75-80% making it difficult to control PPR in Ngorongoro (Karimuribo *et al.*, 2011).

1.2 Problem Statement and Justification

Peste des petits ruminants is relatively new in Tanzania and the molecular epidemiology of the PPRV is poorly understood. Its high rate of spread, morbiditics and mortalities impose major small ruminant production constraints in arid and semi-arid areas where small ruminants are the principle source of income (Mtenga *et al.*, 1986). In Tanzania, the livestock population includes nearly 22.6 million domestic small ruminants. Of these, goats are the most abundant (about 15.6 million) and the majority of the livestock population is owned by pastoralists and agro-pastoralists in the northern districts. Mortalities due to PPR in small ruminants lead to economic losses and food insecurity. Therefore, generating any evidence informing the molecular epidemiology of the virus is important for better understanding of the disease and designing and implementing control strategies.

1.3 Research Objectives

1.3.1 General objective

To detect and perform molecular characterization of PPRV in goats of Ngorongoro District, Arusha Region

1.3.2 Specific objectives

i) To detect PPRV by RT-PCR from samples collected from clinically sick and dead goats of Ngorongoro District

ii) To characterize PPRV and identify PPRV lineage circulating in Ngorongoro District and

iii) To describe epidemiological, clinical and pathological findings in PPR suspected cases in goats of Ngorongoro District

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Aetiology and Molecular Epidemiology

Peste des petits ruminants has been classified under family *Paramyxoviridae*, order Mononegavirales and genus *Morbillivirus* (Tober *et al.*, 1998). Like other *morbilliviruses*, PPRV is fragile, therefore, cannot survive for long time outside the host. The half life for this virus has been estimated to be 2.2 minutes at 56° C and 3.3 hours at 37° C (Rossiter and Taylor, 1994). The virus is an enveloped pleomorphic particle, single stranded RNA, with an approximate size of 16 kb long with negative polarity (Haas *et al.*, 1995).

The genome is composed of 15,948 nucleotides, the longest of all mobillivirus genomes sequenced so far. This genomic RNA is wrapped by the nucleoprotein (N) to form the nucleocapsid into which are associated two other viral proteins: the phosphoprotein (P) and the large protein (L). The phosphoprotein is the cofactor of L, the viral RNA dependent RNA polymerase (RdRp). The viral envelope is composed of three proteins namely; the matrix protein (M) which is located inside the envelope and serves as a link between the nucleocapsid and the two external viral proteins, the fusion protein (F) and the haemmagglutinin (H). The M protein plays an important role in ensuring efficient incorporation of nucleocapsids into virions during the virus budding process. The H protein allows the virus to bind to the cell receptor during the first step of the viral infection process while the F protein is responsible for virus entry and uncoating of the genome. Both the F and H proteins are very important in the induction of protective host immune response against the virus. However, N is the most abundant and also the most immunogenic among PPRV proteins which does not induce protective immunity against the virus. Diallo *et al.* (2007) reported this gene to have been used in the development of

molecular diagnostic tests. Partial sequencing of N gene revealed some small variations between PPRV strains, thus, grouping the virus into four lineages (I-IV) that better reflects their geographical origins than the variations of genes of the external glycoproteins F and H.

2.2 Disease Distribution

Peste des petits ruminants was first described in West Africa in 1940s (Gargadennec and Lalanne, 1942). Since then, the virus has spread to other countries as well. Peste des petits ruminants has been reported in Southern Asia, Near East, Arabian Peninsula and in recent years outbreaks have been reported in Turkey (2000), Tibet China (2007), Morocco (2008), Tanzania (2008) and Uganda in 2007 (Luka *et al.*, 2011). In Tanzania, PPR outbreak was first reported in 2008 in northern parts of the country (Swai *et al.*, 2009) and is believed to have been introduced from Kenya. However, a retrospective study performed using archived samples shows that PPR may have been introduced into Tanzania as back as 2004 (Karimuribo *et al.*, 2011). Muse *et al.*, (2012) confirmed the presence of PPR in the southern parts of Tanzania affecting sheep and goats of Newala and Tandahimba districts in Mtwara Region.

2.3 Transmission

Peste des petit ruminants virus is highly contagious and is transmitted by direct contact involving secretions (like nasal and ocular secretions, saliva) or excretions (like urine and feces) from infected animals to healthy animals in close proximity especially when grazing and in water points (Ezeibe *et al.*, 2008). Furthermore, the existence of sylvatic reservoirs for PPRV has been reported with infections and deaths in captive wild ungulates from several species (Kinne *et al.*, 2010).

2.4 Clinical Manifestations

Clinically, PPR is characterized by sudden onset of a febrile illness with rectal temperature recorded as high as 41° C. Purulent discharges from eyes, nose and mouth with sores in the mouth, with or without scabs or nodules around the mouth. Pneumonia, diarrhoea and significant high mortalities are among the major features for suspicion of PPR infection (FAO, 1999). Muse *et al.*, (2012) reported nodular lesions all over the body in some goats that tested positive to PPR in the southern Tanzania.

2.5 Pathology

At necropsy, the lesions are very similar to those observed in cattle affected with rinderpest, except prominent crusty scabs along the outer lips of the mouth and severe interstitial pneumonia frequently occur with PPR. Erosive lesions may extend from the mouth to the reticulo–rumen junction. Characteristic linear haemorrhages or zebra stripes occur in the large intestine, commonly at the caeco-colic junction, but they are not a consistent finding; necrotic or haemorrhagic enteritis is usually present. Lymph nodes are enlarged, the spleen may show necrotic lesions and there is an apical pneumonia (OIE, 2011; Muse *et al.*, 2012).

2.6 Diagnosis

The clinical signs and post-mortem lesions are highly suggestive in acute and per-acute cases. Virus isolation and characterization can be done by collecting blood in heparin or EDTA anticoagulants and tissues sections obtained from lesions of lymph nodes, tonsils, spleen and lungs. In live animals, however, swabs of ocular and nasal discharges and oral lesions can be used.

Peste des petits ruminants antibodies can be detected in serum by using competitive enzyme-linked immunosorbent assay (cELISA) that was developed by Anderson and

McKay (1991). Virus neutralization tests can also be performed (Singh *et al.*, 2009). Couacy-Hymann *et al.* (2002) reported rapid and sensitive molecular techniques such as RT-PCR used to detect the nucleic acid of the virus.

2.7 Prevention and Control

Control of PPR outbreaks relies on movement control such as quarantine, efficient disease reporting, rapid response to outbreaks (Saravanan *et al.*, 2010) coupled with the use of vaccine by doing a roll back vaccination (Nyamweya *et al.*, 2009). However, proper disposal of carcass and contact fomites, decontamination and restriction on importation of sheep and goats from affected areas can help to prevent from PPR infection (Chauhan *et al.*, 2009). However, vaccination has remained to be the only feasible option because of the inability to afford the zoo-sanitary control measures in most developing countries (Luka *et al.*, 2011). Rossiter and James (1989) recommended a minimum of 75-80% vaccination coverage for the effective control of rinderpest which can also be employed for PPR.

2.8 Socio-economic impacts of PPR

Peste des petits ruminants like any other transboundary animal disease, have major economic implications both through the public and private cost of outbreaks (FAO, 2004). During 2007-2008 PPR outbreaks in Kenya, the Government estimated annual losses of over US$ 13 million (Kihu *et al.*, 2010). Studies conducted in Turkana, Kenya show that PPR outbreak contributed to food insecurity and reduced income particularly in livestock based societies. In early 2010, local population of over 13.5 million goats and over 3.5 million sheep in Tanzania were threatened by PPR. Maasai pastoralist in the Hai district in Kilimanjaro region reported a loss of about 75 percent of their animals due to the high mortalities during the 2007-2008 PPR outbreaks (FAO, 2010).

Major areas where economic impacts of disease can be assessed include production, trade, price and market effects, food security and nutrition, financial costs as well as health and environment (Otte *et al.*, 2004).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was conducted within Ngorongoro District in Meshili village within Ngorongoro Conservation Area Authority (NCAA) and Piyaya and Malambo villages within Loliondo Game Controlled Area (LGCA) (Fig. 1). Ngorongoro District is located in the Northern Tanzania with a population of 174, 278 people (NBS, 2013). According to the District Veterinary Office reports of 2012, there were approximately 400,000 sheep and 300 000 goats. The district is bordered by the Serengeti National Park to the west, the Kenya international border to the north, Monduli District to the east and Karatu District to the south (Fig. 1). Ngorongoro District was selected because it has been experiencing several outbreaks since the official confirmation of PPR in 2008 despite the vaccination that has been carried out. The district is considered to be a risk area because it borders Kenya, the neighbouring country believed to have introduced the disease in northern Tanzania and it forms part of the northern transboundary animal movement route.

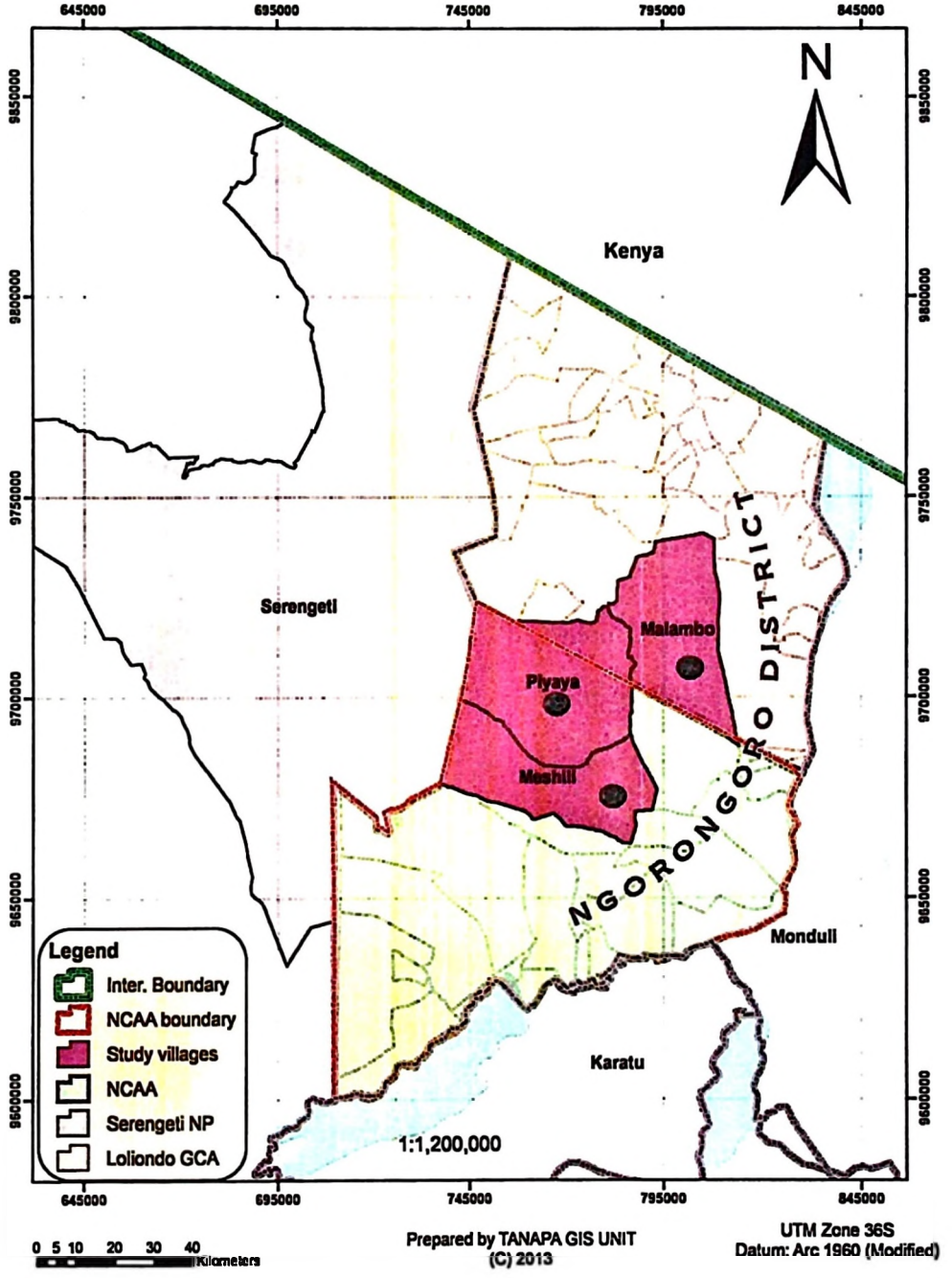

Figure 1: Map of Tanzania showing the Ngorongoro District, its surrounding areas and the villages where this study was conducted (shown with a black dot). NCAA, Ngorongoro Conservation Area Authority; GCA, Game Controlled Area.

3.2 Animals and Sample Collection

A cross sectional study design was used where farms (bomas) and animals sampled were purposively selected relying upon the reports of deaths and clinical disease from a livestock field officer of the responsible ward. In clinically affected flocks, animals were physically examined and data including age, sex, temperature and clinical manifestation were recorded. Total of 26 goats were examined and samples were collected. Samples that were collected include 6 tissues (spleen, liver, tongue, intestines, mesenteric lymph nodes and lung), 20 swabs (lacrimal, nasal and oral), 27 whole blood (in plain, EDTA and heparin tubes) and 24 sera. Swabs were put in the viral transport media and immediately stored at -100° C in a dry shipper charged with liquid nitrogen. Serum was harvested from plain tubes by using a sterile pipette and transferred into the cryovials and stored in the dry shipper. Postmortem examination was performed in dead animals and postmortem findings were recorded. All samples in cryovials were put in the secondary container and stored in the dry shipper (at -100 °C). The samples were transported to the Genome Sciences Centre, Faculty of Veterinary Medicine at the Sokoine University of Agriculture for laboratory analyses. In the laboratory, the samples were stored at -80° C ultra-low temperature freezer until when they were processed.

3.3 Molecular detection of PPRV by RT-PCR

3.3.1 Preparation of mononuclear cells from blood

One milliliter of the sample was put into each tubes containing 500 µL of histopaque solution. A mixture was centrifuged at 6000 *g* at 4° C for 30 min. The upper layer containing plasma was discarded and the contents in the interface (mononuclear cells) were collected by using a pipette and transferred into another tube and a phosphate buffer saline (PBS) was added and incubated at room temperature (25° C). A total of 10 blood samples were prepared.

3.3. 2 Processing of swabs

One milliliter of PBS was added into each cryovials containing swabs, vortexed for 15 seconds and incubated at room temperature (25° C) for 10 min. The supernatant was collected and put into new Eppendorf tubes. A total of 12 samples from swabs were prepared.

3.3.3 Processing of tissues

Spleen, liver, tongue, intestines, mesenteric lymph nodes and lung samples from individual goats were pooled, macerated by a sterile scapel blade and homogenized. Approximately 250 mg of tissue sample was homogenized in 2.5 ml of Gibco® F-12 Nutrient Mixture (Invitrogen, Carlsbad, USA) to make a 10% tissue suspension. The tissue suspension was then centrifuged at 8000 x g for five minutes at room temperature and stored at -80° C until RNA extraction.

3.3.4 RNA extraction

RNA extractions was done by a spin protocol technique using QIAamp Viral RNA Mini extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 560 µL of a prepared Buffer AVL containing carrier RNA was pipetted into microcentrifuge tubes, then, 140 µL of the sample was added into each respective tube. The solution was mixed by pulse-vortexing for 15 seconds and incubated at room temperature (15–25° C) for 10 minutes. Fast spin was done to remove drops from the inside of the lid. Five hundred and sixty micro liters of absolute ethanol was added to the sample, and mixed by pulse-vortexing for 15 seconds. Fast spin was again done to remove drops from inside the lid. Futhermore, 630 µL of the resulting solution was carefully applied to the QIAamp Mini column and centrifuged at 6000 *g* for 1 minute. The QIAamp Mini column was placed into a clean 2 ml collection tubes while discarding the tubes

containing the filtrate (this step was repeated twice). Five hundred microlitres of Buffer AW1 was added and placed in the QIAamp Mini column and centrifuged at 6000 *g* for 1 minute. QIAamp Mini column was placed in a clean 2 ml collection tubes and the tubes containing the filtrate were discarded. Then 500 µL of Buffer AW2 was carefully added in the QIAamp Mini column and centrifuged at full speed 20 000 *g* for 3 minutes. The QIAamp Mini column was then placed in a clean 1.5 ml microcentrifuge tube and the tubes with filtrates were discarded. Sixty microlitres of elution buffer was carefully added in the QIAamp Mini column and incubated at room temperature for 1 minute. Then centrifugation at 6000 *g* for 1 minute was done in order to concentrate the RNA product. The RNA products were stored at -20° C in the deep freezer.

3.3.5 RT-PCR

The detection of PPRV F and N genes was performed using two sets of primers F1/F2 and NP3/NP4, respectively. The expected band size after amplification of the F and N genes was of 448bp and 351bp, respectively. A reverse transcription polymerase chain reaction (RT-PCR) was carried out using GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, USA). The master mix was prepared using the AgPath-ID one-step RT-PCR kit (Applied Biosystems, Courtaboeuf, France) with PPRV specific primers from Eurogentec (Liège, Belgium) (Tables 1 and 2). A copy DNA (cDNA) was synthesized from the positive samples to make the RNA stable for storage and future use. A reaction mixture was prepared containing; 2X RT-PCR buffer, random hexamer, 25X RT-PCR enzyme mix, nuclease free water and respective RNA sample (Table 1). The condition of the thermocycler during cDNA synthesis using RT-PCR was set at 42° C for 45 minutes as the denaturation condition, 94°C for 5 minutes as an annealing temperature and 20° C for ∞ (infinite). DNA amplification by PCR was carried out using NP3/NP4 and F1/F2 primers and cycling conditions shown in Fig. 2

Table 1: RT-PCR master mix components

No.	Component	Volume (µl)
1.	2X RT-PCR Buffer containing dNTPs	12.5
2.	10µM Forward primer (NP3 or F1)	1.0
3.	10µM Reverse primer (NP4 or F2)	1.0
4.	Nuclease-free water	9.0
5.	25X RT-PCR enzyme mix containing a reverse transcriptase and DNA polymerase	0.5
6.	Extracted RNA template	1.0
	Total volume per reaction	**25.0**

Table 2: List of primers used for detection of PPRV genome

No.	Name	Gene	Location	Sequence	Amplicon size	Reference
1.	NP3	Nucleoprotein	1232-1255	5'- TCTCGGAAATCGCCTCACAGACTG -3'	351bp	Ularamu *et al.*, 2012; Couacy-Hymann *et al*, 2002
2.	NP4	Nucleoprotein	1583-1560	5'- CCTCCTCCTGGTCCTCCAGAATCT -3'	351bp	
3.	PPRV-F-F	Fusion protein	777-801	5'- GAGACTGAGTTTGTGACCTACAAGC -3'	322bp	Esmaelizad *et al.*, 2011; Forsyth and Barrett, 1995
4.	PPRV-F-R	Fusion protein	1124-1148	5'- ATCACAGTGTTAAAGCCTGTAGAGG -3'	322bp	

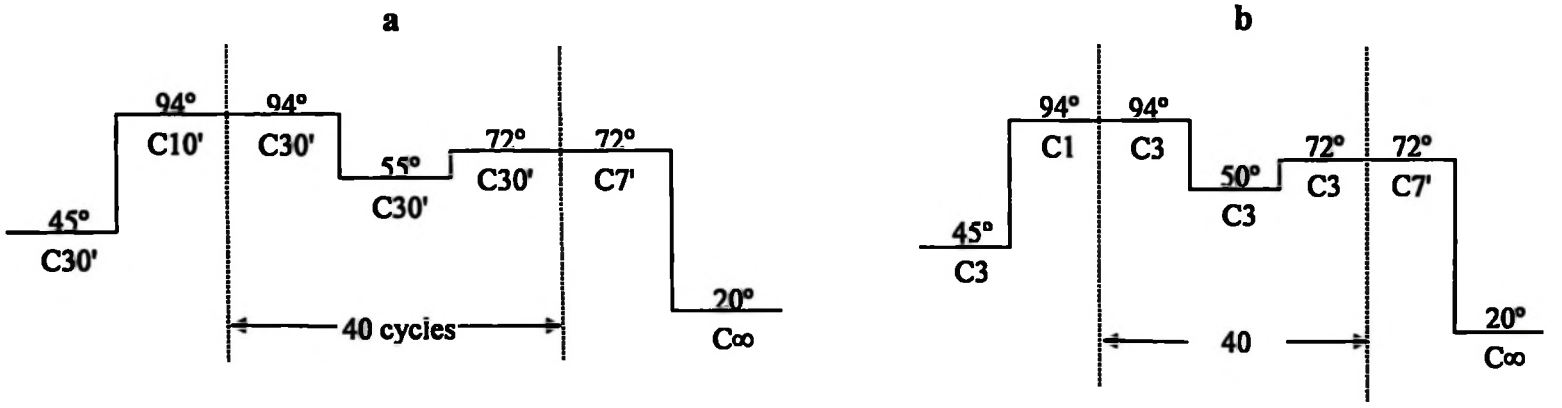

Figure 2: Reverse transcription polymerase chain reaction cycling conditions for the amplification of (a) N gene using NP3/NP4 primers and (b) F gene using PPRV F-F/R primers where ' represents minutes, " represents seconds and ∞ represents infinity.

3.3.6 DNA visualization

A 1.5% agarose stained with GelRed nucleic acid stain (Phenix Research Products, Candler, USA) in TAE buffer (SERVA Electrophoresis, Heidelberg, Germany) was used during gel electrophoresis to separate and visualize the expected bands of DNA. Each well was loaded with 5 µl of the PCR product and 1 µl of blue/orange 6X DNA loading dye (Promega, Madison, USA). Samples were separated along by using 100bp DNA ladder to determine size of the bands. An electrophoretic cell was run at 100 mA for 30 minutes. The gel was carefully removed from the casting tray and put onto the ultraviolet fluorescent light transilluminator (Sigma-Alderich, St. Louis, USA). The bands were visualized by the aid of an eye protector and recorded using a digital camera.

3.4 Nucleotide Sequencing

Polymerase chain reaction (PCR) products were treated with exonuclease I and alkaline phosphatase before they were sequenced directly using Big Dye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA). Alternatively, PCR fragments were purified from agarose gels using a NucleoSpin gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany). The gel purified PCR fragments were cloned in a TOPO-T/A plasmid vector (pCR4-TOPO) (Invitrogen, Carlsbad, CA). Afterwards, dideoxynucleotide cycle sequencing reaction was performed using standard M13 forward (5'-GTAAAACGACGGCCAG-3') and M13 reverse (5'-CAGGAAACAGCTATGAC-3') primers for sequencing of PPRV partial N gene inserts in the TOPO-T/A plasmid vectors using Big Dye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA). Sequenced PCR products were purified by ethanol precipitation and separated on a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA).

3.5 Data Analysis

Microsoft Office Excel 2007 programme was used to calculate frequencies of PPR positive samples. The sequences were edited by BioEdit® software and aligned using

MEGA 5.05®. The BLAST programme was used to search in the GenBank for homologous gene sequences in the NCBI database. A phylogenetic tree was constructed by the aid of MEGA 5.05® software using the neighbour-joining method employing the Kimura-2-option (Tamura *et al.*, 2011).

CHAPTER FOUR

4.0 RESULTS

4.1 Epidemiological, Clinical and Gross Pathological Findings

According to personal communication with farmers, most farms (bomas) and flocks that were affected had no history of vaccination against PPR. In the flocks that had active cases, clinical signs varied considerably. Major signs recorded include fever where the rectal temperature ranging from 38.9° C to 41.0° C with an average of 40.0° C, purulent oculonasal discharges that caused matting of eye lids in some animals, diarrhea, loss of appetite, dullness and labored breathing (Fig. 3). Ulcerations were also observed surrounding the mouth, nose, gums, tongue and buccal cavity. It was also observed that, young animals were showing more severe clinical signs than the adults. Postmortem results revealed swollen and congested lung (pneumonia) especially affecting the cranial lobes, oedematous and congested retropharyngeal and mesenteric lymph nodes and linear haemorrhages in the mucosa of intestines with the formation of a characteristic pattern (Fig. 4). Summary of animal biodata, clinical signs observed, gross pathology and samples collected is shown in Appendix I.

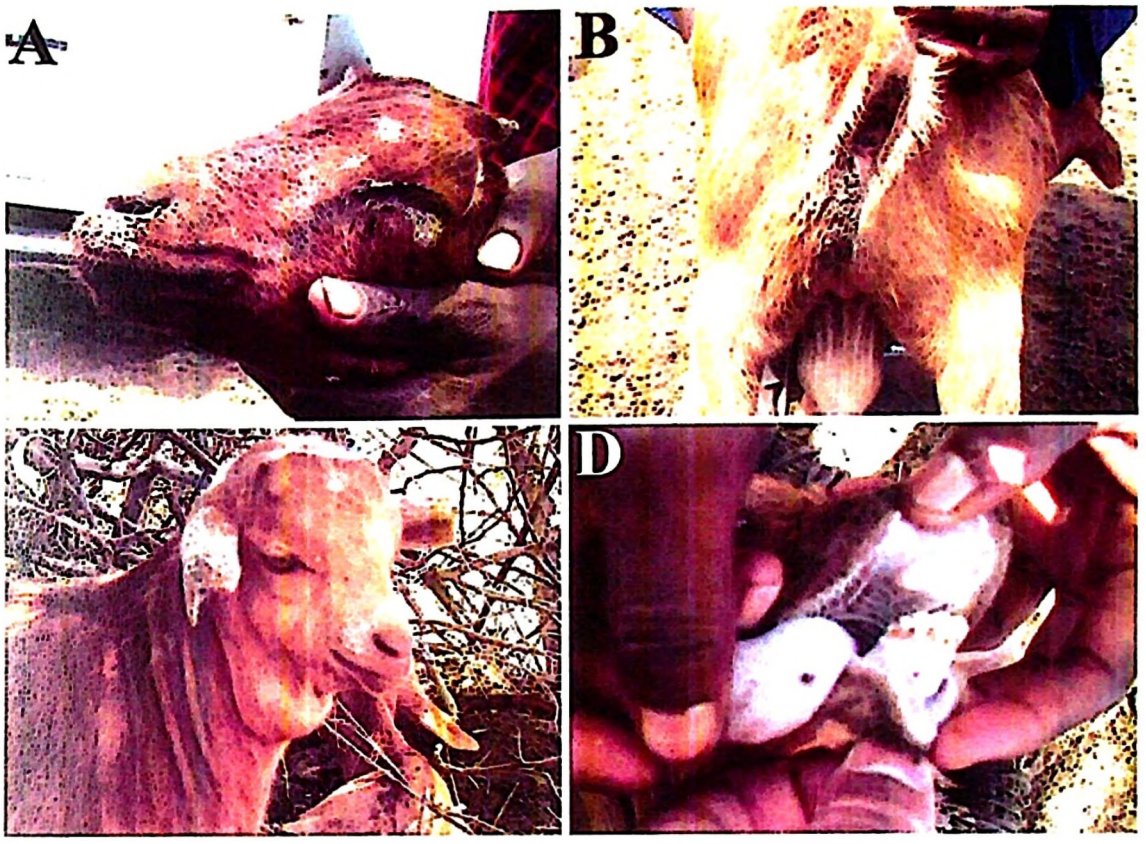

Figure 3: Clinical presentation showing oculonasal discharges and matting of eye lids (A), diarrhea shown by soiling of the perineum (B), submandibular oedema (C) and sores and nodules on the gums and tongue (D).

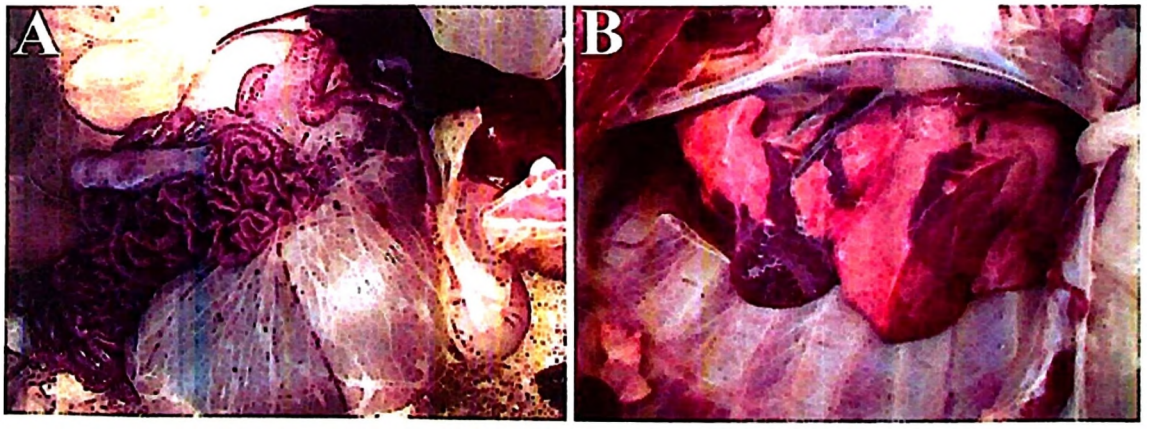

Figure 4: Postmortem findings showing hemorrhages in the intestine (A) and pneumonia (B).

4.2 RT-PCR results

Twenty eight samples were subjected to RT-PCR, of which seven samples (25%) tested positive for PPR using the NP3/NP4 primers resulting into a 351 bp PCR bands. None of the samples tested was positive when primers F1/F2 were used. All the sample types, that is whole blood, swabs and tissues tested positive. Moreover, 33.3%, 30.0% and 16.7% of tissues, whole blood and swabs were positive, respectively (Table 3). The results also show that there are more positives in adults (36.4%) as compared to kids (20.0%), more so, there are more female positives (42.7%) than males (8.3%).

Table 3: Distribution of RT-PCR for N gene results with age, sex and type of sample

Category		Number of goats tested	PPRV positive goats (%)
Age	Adults	11	4 (36.4)
	Kids	15	3 (20.0)
Sex	Males	12	1 (8.3)
	Females	14	6 (42.7)
Type of sample	Whole blood (EDTA)	10	3 (30.0)
	Tissues	6	2 (33.3)
	Swabs	12	2 (16.7)

4.3 PPRV Nucleotide Sequences and Phylogenetic Tree

The three partial N gene sequences obtained were aligned using CLUSTAL-W implemented in MEGA 5.05® and found to be 100% identical. The nucleotide sequence of the N gene of PPRV collected in goats from Ngorongoro (Tanzania/Ngorongoro/2013) obtained in this study was submitted at GenBank and assigned accession number KF939644. The BLAST programme was used to search in the GenBank for homologous gene sequences in the NCBI database with one of the sequences (a consensus sequence),

Tanzania/Ngorongoro/2013 (Table 4). The analysis of Tanzania/Ngorongoro/2013 sequence revealed closest nucleotide identities of 95% with previously characterized isolates from Ethiopia with accession number DQ840183 (Table 4 and Fig. 5).

Table 4: BLAST results showing homologous gene sequences at the Genbank database

SN	Description	Maximum score	Query cover (%)	Percentage identities	Accession number
1	Meilik 72	566	100	96	JN647693
2	MBLNUC	566	100	96	L39878
3	Ethiopia 1994	490	86	96	JN647699
4	Ethiopia	405	72	95	DQ840183
5	Sudan Meilik/72	405	72	95	DQ840159
6	Sudan Sinar/72	405	72	95	DQ840158
7	Ethiopia 1994	366	72	93	DQ840175
8	DORCAS 87	483	100	91	JN647695
9	TR/ANTALYA (Ibrad) 2012/1308N	372	100	86	KF478927
10	TR/KONYA (Kulu) 2013/332N	372	100	86	KF478926
11	TR/KAHRAMANMARAS/2011/48	372	100	86	JQ519916
12	Kurdistan/2011	372	100	86	JF969755
13	TR/NIGDE (Ulukisla)/2011/1223N	366	100	85	KF478931
14	TR/HATAY/2011/45	366	100	85	JQ388658
15	TR/HAYAT/2011/44	366	100	85	JQ388656
16	BD-PPR-DHAKA-1/2010	363	99	85	JQ612706
17	Shiraz1	361	100	85	KC534492
18	Shiraz101	361	100	85	KC152953
19	TR/TEKIRDAG/2011/4	361	100	85	JQ519959
20	TR/SANLIURFA/2011/51	361	100	85	JQ519951

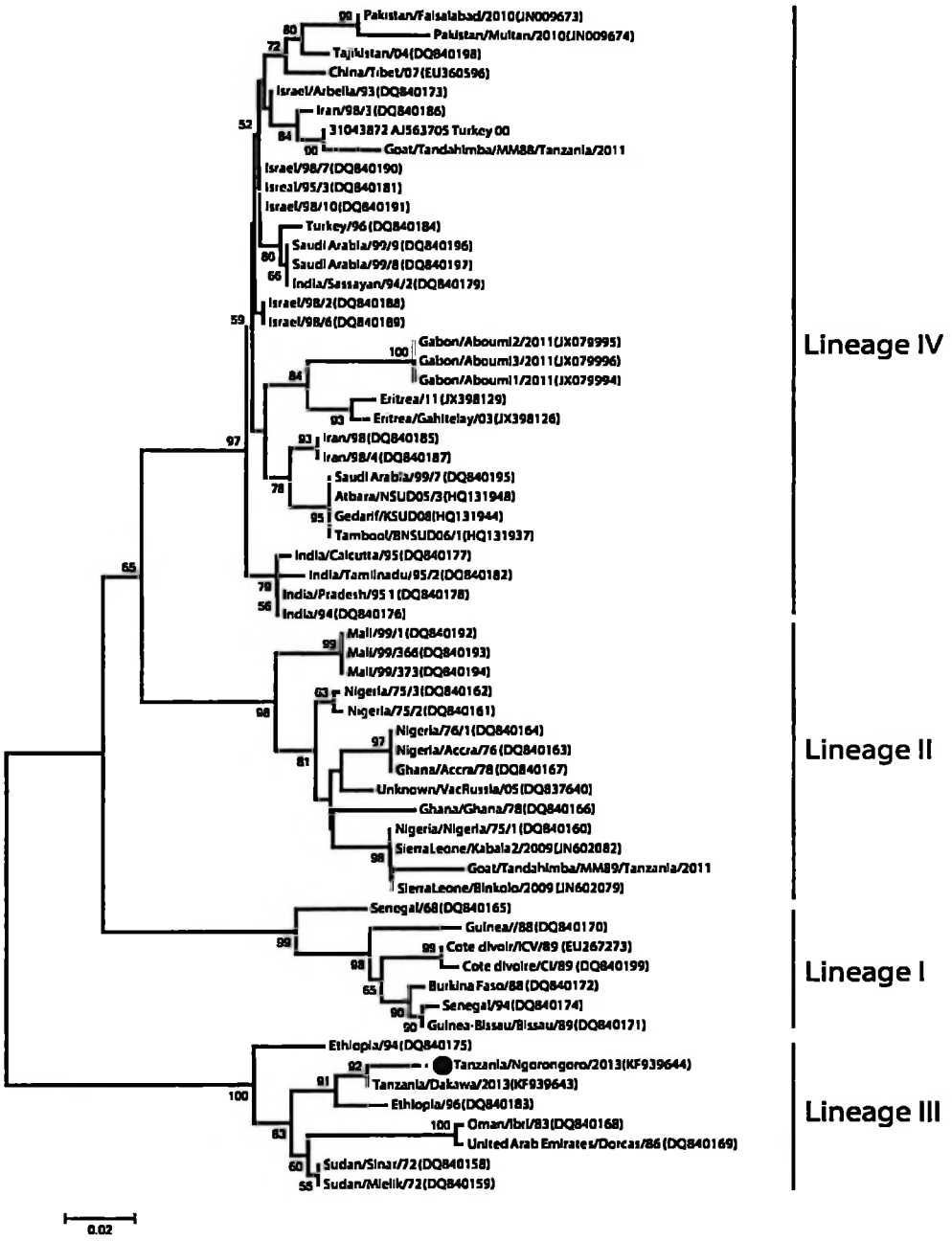

Figure 5: Phylogenetic tree of PPR viruses based on the N gene constructed using the neighbour-joining method inferred following 1000 bootstrap replications in MEGA 5.05® software. Isolates of PPRV used to construct this tree are listed in Appendix 2. The PPRV strain obtained in this study, Tanzania/Ngorongoro/2013 (accession number KF939644), is marked with a round black dot.

CHAPTER FIVE

5.0 DISCUSSION

Clinical signs and gross pathological findings from the observed animals in this study were highly suggestive of presence of the PPR. Animals had erosions on the mucous membrane of the buccal cavity and tongue, profuse diarrhea (pasting of the perineum) and elevated rectal temperatures above 40 °C. These findings concur with what has been reported by Aktas *et al.* (2011). The pathology of lungs, intestines and their associated lymph nodes are similar to findings reported by Chauhan *et al.* (2009) as common features of PPR. However, although clinical signs and gross pathological findings may be typical and highly suggestive of PPRV infection, alone they cannot be used to confirm 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 by animals that do not show clinical signs. The results for females being more affected (42.7%) than males (8.3%) are in agreement with what have been also reported by Waret-Szkuta *et al.*, (2008) where they observed sex related differences in seroprevalence in goats where females were highly susceptible than males.

Molecular detection of PPRV based on the F gene developed by Forsyth and Barrett (1995) and the N gene developed by Couacy-Hymann *et al.* (2002) is apparently a popular PCR tool for detection of the PPRV genome. In this study, PPRV was confirmed by RT-PCR using primers specific for N gene (NP3/NP4) but not the F gene (F1/F2). Kerur *et al.* (2008) and Bhuiyan *et al.*, (2012) also report that N gene based primers were more sensitive than F gene based primers. The lower sensitivity with F gene specific primers might be attributed to the nature of PPRV genome which shows higher rate of transcription of N-gene than F-gene in what is called the transcription gradient. Forsyth

and Barrett (1995) found that the target for PCR primers was F gene, one of the external viral proteins. Other results (Steinhauer and Holland, 1986) have indicated that the N gene that codes for an internal structural protein and mRNAs of N gene are the most abundant transcripts of the virus, making it attractive for development of a highly sensitive assay.

The virus was detected from all sample types including swabs (nasal, oral and ocular), whole blood and tissues from lymph nodes, intestines, tongue, liver, spleen and lungs. Results being positive especially from whole blood and swabs provide an important insight as swabs and blood are easy to obtain in live animals thus becomes an important clinical sample for diagnostic purposes. Luka *et al.* (2011) also noted that the use of swabs in detection of PPRV is important in countries where the disease mortality rates are relatively low to do necropsy. Forsyth and Barrett (1995) reported that swabs taken from infected animals in the field not only provide a suitable source of viral RNA, but are also not subject to the same storage and transport problems associated with tissue samples.

Furthermore, to assess the genetic similarity, previously characterized PPRV N gene nucleotide sequences at GenBank were analyzed along with the N gene nucleotide sequence obtained in this study i.e Tanzania Ngorongoro 2013. The PPRV from Ngorongoro showed a 95% nucleotide identity with the isolate from Ethiopia. Phylogenetic analysis clustered the Ngorongoro PPRV strain into lineage III. Apparently, the molecular epidemiology of the PPRV in Tanzania is poorly understood, thus its sources still remain questionable. However, due to international borders being porous for animal movements and trade, it is possible that the virus might have been introduced from the neighboring countries. It is possible that PPR was introduced into Tanzania from Sudan through Kenya as previously suggested by Kivaria *et al.* (2013).

The results obtained in the present study indicate that the PPRV circulating in Ngorongoro clusters into lineage III. However, the available vaccine used for protecting sheep and goats in African countries is developed from Nigeria 75/1 strain, which clusters under lineage II. It is worthy investigating whether lineage II vaccines protect goats and sheep against other lineages of PPRV. This study was undertaken within the Ngorongoro Conservation Area and Loliondo Controlled Game Area where livestock graze along with wild animals. It is worthy investigating whether wild animals act as reservoirs of PPRV, a fact that may explain the persistent circulation of PPR in northern Tanzania.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Peste des petits ruminants was present and confirmed to be highly associated with the recent outbreak of the disease that caused acute deaths in sheep and goats in Ngorongoro area. However, outbreaks occurred mainly in the flocks and individuals that were previously not vaccinated. The study also shows that by using RT-PCR, PPRV can be detected from tissues, whole blood and swabs. Peste des petit ruminants virus (PPRV) circulating in Ngorongoro is clustered in linage III which is different from the vaccine strain which falls under linage II. Although primers specific for F gene (F1/F2) did not detect any positive result, PCR NP3/NP4 primers targeting the N gene could detect PPRV, indicating that it is desirable to use more than one set of primers for the efficient amplification and detection of PPRV. Primers targeting F and N genes are the most suitable as have been recommended by Kerur *et al.* (2008).

6.2 Recommendations

Based on findings from this study, it is desirable to use more than one set of primers for the efficient amplification and detection of PPR viruses. The continued circulation of PPRV in Ngorongoro District warrants further studies in order to determine if wild animals serve as reservoirs of PPR. Furthermore, more studies on the epidemiology of PPR are recommended to collect more data for effective control measures including characterizing the strains that can be used for development of vaccines using local strains. In addition, detection and genotyping of PPRV circulating in Kenya needs to be performed in order to map the spread of PPRV between Ethiopia and Tanzania.

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APPENDICES

Appendix 1: Clinicopathological distribution and samples collected

Species	Sex	Age	Clinical signs	PM lesions	Samples	Sample ID	Remarks
Caprine	M	Kid	41°C, purulent oculonasal discharges	Pneumonia	Whole blood, serum, lungs, colon, mesenteric L/N, mouth, tongue and spleen	T1	Sacrified
Caprine	F	Ad	41°C, purulent nasal secretions	NA	Whole blood serum	B2	PPR suspect
Caprine	F	Kid	40°C, dullness	NA	Whole blood, serum	B3	PPR suspect
Caprine	F	Ad	40.8°C, mucopurulent nasal discharges	NA	Whole blood, serum	B4	PPR suspect
Caprine	F	Kid	40.8°C	NA	Whole blood, serum	B5	PPR suspect
Caprine	F	Kid	39.7°C, diarrhea, mucopurulent nasal discharge, emaciation	NA	Whole blood, serum, swab	S6	PPR suspect
Caprine	F	Kid	39.2°C, diarrhea, lacrimation, emaciation	NA	Whole blood, swab	S7	PPR suspect
Caprine	M	Kid	39.7°C, diarrhea, lacrimation, oral lesions	NA	Whole blood, serum, swab	S8	PPR suspect
Caprine	M	Kid	39.1°C, diarrhea	NA	Whole blood, serum	S9	PPR suspect
Caprine	M	Kid	NA	Purulent oculonasal discharges, pneumonia	Spleen, lungs, mesenteric L/N, intestines, nasal lesions	T11	Dead, PPR suspect
Caprine	M	Kid	39.2°C, diarrhea	NA	Whole blood, serum	B12	PPR suspect
Caprine	F	Ad	40.9°C, purulent oculonasal discharges, labored breathing	NA	Whole blood, serum, nasal/oral swabs	S14, T14	Sacrified, PPR suspect
Caprine	M	Ad	40.8°C, labored breathing, purulent oculonasal secretions, mouth and nasal lesions	NA	Whole blood, serum, swabs	S15	PPR suspect
Caprine	F	Kid	40.0°C, diarrhea, oculonasal discharges	NA	Whole blood, serum, swabs	S16	PPR suspect
Caprine	M	Ad	38.9°C, diarrhea	NA	Whole blood, serum, swabs	S17	Normal
Caprine	F	Kid	40.0°C, diarrhea, matting of eye lids	NA	Whole blood, serum, swabs	S18	PPR suspect
Caprine	F	Ad	39.6°C, diarrhea	NA	Whole blood, serum	S19	Normal

Caprine	F	Ad	40.3°C, diarrhea, purulent oculonasal discharges, labored breathing	NA	Whole blood, serum, swabs	S20	PPR suspect
Caprine	M	Ad	40.4°C, purulent nasal secretions, mouth lesions	NA	Whole blood, serum swabs	S21	PPR suspect
Caprine	F	Kid	VIC-Arusha	NA	Whole blood	BV1	PPR suspect
Caprine	M	Kid	VIC Arusha	NA	Whole blood	BV2	PPR suspect
Caprine	M	Ad	VIC Arusha	NA	Whole blood	BV3	PPR suspect
Caprine	M	Ad	VIC Arusha	NA	Whole blood	BV4	PPR suspect
Caprine	F	Kid	VIC Arusha	-	Tissues	TV5	PPR suspect
Caprine	F	Kid	VIC Arusha	-	Tissue	TV6	PPR suspect

Appendix 2: List of all PPRV isolates used to construct the phylogenetic tree

Name of isolate	Year isolated	Country of origin	Accession No.	Reference
Pak_Faisalabad/2010	2010	Pakistan	JN009673	Maganga *et al.*, 2013
Multan	2010	Pakistan	JN009674	Maganga *et al.*, 2013
04	2004	Tajikistan	DQ840198	Maganga *et al.*, 2013
China/Tibet/0701	2007	China	EU360596	Munir *et al.*, 2012a
Arbella/93	1993	Israel	DQ840173	Maganga *et al.*, 2013
98/3	1998	Iran	DQ840186	Munir *et al.*, 2012a
Turkey/00	2000	Turkey	AJ563705	Maganga *et al.*, 2013
Tandahimba/MM88	2011	Tanzania		Misinzo *et al.*, 2013
98/7	1998	Israel	DQ840190	Munir *et al.*, 2012a
95/3	1995	Israel	DQ840181	Munir *et al.*, 2012a
98/10	1998	Israel	DQ840191	Munir *et al.*, 2012a
96	1996	Turkey	DQ840184	Maganga *et al.*, 2013
99/9	1999	Saudi Arabia	DQ840196	Munir *et al.*, 2012a
99/8	1999	Saudi Arabia	DQ840197	Munir *et al.*, 2012a
Sassayan94/2	1994	India	DQ840179	Maganga *et al.*, 2013
98/2	1998	Israel	DQ840188	Maganga *et al.*, 2013
98/6	1998	Israel	DQ840189	Maganga *et al.*, 2013
Gabon Aboumi2 2011	2011	Gabon	JX079995	Maganga *et al.*, 2013
Gabon Aboumi3 2011	2011	Gabon	JX079996	Maganga *et al.*, 2013
Gabon Aboumi1 2011	2011	Gabon	JX079994	Maganga *et al.*, 2013
Eritrea/11	2011	Eritrea	JX398129	Cosseddu *et al.*, 2013
Eritrea/Gahitelay/03	2003	Eritrea	JX398126	Cosseddu *et al.*, 2013
98	1998	Iran	DQ840185	Maganga *et al.*, 2013
98/4	1998	Iran	DQ840187	Maganga *et al.*, 2013
99/7	1999	Saudi Arabia	DQ840195	Maganga *et al.*, 2013
Atbara NSUD05/03	2005	Atbara	HQ131948	Munir *et al.*, 2012a
Gedarif KSUD08	2008	Gedarif	HQ131944	Munir *et al.*, 2012a
Tambool-BNSUD06-1	2006	Tambool	HQ131937	Munir *et al.*, 2012a
Calcutta/95	1995	India	DQ840177	Munir *et al.*, 2012a
Pradesh_95	1995	India	DQ840178	Munir *et al.*, 2012a
94	1994	India	DQ840176	Munir *et al.*, 2012a
99/1	1999	Mali	DQ840192	Luka et al., 2011
99/366	1999	Mali	DQ840193	Luka *et al.*, 2011
99/373	1999	Mali	DQ840194	Luka *et al.*, 2011
75/3	1975	Nigeria	DQ840162	Luka *et al.*, 2011
75/2	1975	Nigeria	DQ840161	Luka *et al.*, 2011
76/1	1976	Nigeria	DQ840164	Luka *et al.*, 2011
Accra/76	1976	Ghana	DQ840163	Munir *et al.*, 2012a
Accra/78	1978	Ghana	DQ840167	Munir *et al.*, 2012a
Unknown vaccine	2005	Russia	DQ837640	Munir *et al.*, 2012a
78	1978	Ghana	DQ840166	Munir *et al.*, 2012a
75/1	1975	Nigeria	DQ840160	Munir *et al.*, 2012a
Sierra Leone/Kabala2/2009	2009	Sierra Leone	JN602082	Munir *et al.*, 2012a

Tandahimba/MM89	2011	Tanzania		Misinzo *et al.*,2013
SierraLeone/Binkolo/ 2009	2009	Sierra Leone	JN602079	Munir *et al.*, 2012a
68	1968	Senegal	DQ840165	Luka *et al.*, 2011
88	1988	Guinea	DQ840170	Luka *et al.*, 2011
ICV89	1989	Cote d'Ivoire	EU267273	Luka *et al.*, 2011
89	1989	Cote d'Ivoire	DQ840199	Luka *et al.*, 2011
88	1988	Burkina Faso	DQ840172	Munir *et al.*, 2012a
94	1994	Senegal	DQ840174	Munir *et al.*, 2012a
89	1989	Guinea-Bissau	DQ840171	Munir *et al.*, 2012a
94	1994	Ethiopia	DQ840175	Munir *et al.*, 2012a
Ngorongoro	2013	Tanzania	KF939644	This study
Dakawa	2013	Tanzania	KF939643	Kgotlele *et al.*, (unpublished)
96	1996	Ethiopia	DQ840183	Munir *et al.*, 2012a
Ibri/83	1983	Oman	DQ840168	Munir *et al.*, 2012a
Dorcas/86	1986	United Arab Emirates	DQ840169	Munir *et al.*, 2012a
Sinar/72	1972	Sudan	DQ840158	Munir *et al.*, 2012a
Mielik/72	1972	Sudan	DQ840159	Munir *et al.*, 2012a