

**SEROPREVALENCE AND GENETIC CHARACTERISATION OF *PESTE
DES PETITS RUMINANTS* VIRUS IN SELECTED AREAS OF TANZANIA**

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ABSTRACT

Peste des petits ruminants (PPR) is an acute, contagious, fatal disease of domestic and wild small ruminants caused by PPR virus (PPRV). A cross-sectional study was conducted to (i) detect PPRV infection, establish the viral lineage and identify the possible source of its introduction and spread in Morogoro district between November 2014 and January 2015 and (ii) determine presence of PPR in selected villages of Ngorongoro district. Clinical samples of six goats from Morogoro urban and Melela village and 252 caprine and ovine sera from villages within and outside Ngorongoro Conservation Area Authority (NCAA) were subjected to molecular and serological analyses, respectively. *Peste des petits ruminants* virus was identified in two goats from Morogoro through amplification of the viral nucleoprotein (N) gene. Phylogenetic analysis of the obtained N gene nucleotide sequences clustered the virus into lineage III and was 99% identical to that of Dakawa reported in 2013. Interviews revealed the source of outbreak in Morogoro urban to be introduction of purchased goats from a livestock market in affected district of Mvomero and interaction of herds in a grazing area was a factor for its spread within the municipality. Overall serology results for samples from Ngorongoro showed 58.33% of animals were infected by PPRV. Percentage of seropositive animals (70.54%) from outside NCAA was significantly higher ($P=0.0004$) than that from within NCAA (48.57%). It is concluded that PPR is not only persistent in previously affected areas, but also is actively spreading to naive areas of Tanzania. It is recommended that veterinary authorities should be vigilant and strict in restricting movement of livestock from affected areas in order to contain PPR.

DECLARATION

I, **Adam Mahamoud Namtimba**, 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 for a degree award in any other institution.

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Date

The declaration is hereby confirmed;

Prof. Gerald Misinzo

(Supervisor)

Date

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DEDICATION

I would like to dedicate this work to my late grandmother Sharifa Chintaka for her kindness, support, encouragement and best wishes in my schooling endeavour.

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LIST OF ABBREVIATIONS AND SYMBOLS

®	registered trade mark
°	degree
A	adenine
ARU	Animal Research Unit
BDSL	Biological Diagnostic Supplies Limited
BLAST	Basic Local Alignment Search Tool
bp	base pair
C	cytosine
C+	positive control serum
C-	negative control serum
Cc	conjugate control
CA	California
Cm	monoclonal antibody control
CAR	Central Republic of Africa
cDNA	complementary DNA
CDV	canine distemper virus
CI	confidence interval
cELISA	competitive enzyme-linked immunosorbent assay
DNA	deoxyribonucleic acid
DRC	Democratic Republic of Congo
E	East
EDTA	ethylenediaminetetraacetic acid

EMPRES	Emergency Prevention System
<i>et al</i>	and others
F	fusion protein
FAO	Food and Agriculture Organization of the United Nations
FVM	Faculty of Veterinary Medicine
G	guanine
<i>g</i>	centrifuge rotor speed
GP	genomic promoter
H	haemagglutinin protein
IFN	interferon
HN	hemagglutinin-neuramidase
HRP	horseradish peroxidase
km	kilometer
km ²	square kilometer
L	polymerase protein
M	matrix protein
M	DNA size marker
MAb	monoclonal antibody
MDBK	Madin-Darby bovine kidney cells
MEGA	Molecular Evolutionary Genetics Analysis
ml	milliliter
MMC	Morogoro Municipal Council
mRNA	messenger ribonucleic acid
MV	measles virus

n	sample size
N	nucleoprotein
N	negative control
NCAA	Ngorongoro Conservation Area Authority
NCBI	National Center for Biotechnology Information
NCS	Non-coding sequence
NP3	nucleoprotein forward primer
NP4	nucleoprotein reverse primer
NSP	non-structural protein
nt	nucleotide
°C	degree Celsius
OD	optical density
OIE	<i>Office International des Epizooties</i>
ORF	open reading frame
P	phosphoprotein
P	positive control
PCR	polymerase chain reaction
pH	degree of acidity or alkalinity
PI	percentage inhibition
PPR	<i>peste des petits ruminants</i>
PPRV	<i>peste des petits ruminants virus</i>
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RNP	ribonucleoprotein

RPV	rinderpest virus
RT-PCR	reverse transcription polymerase chain reaction
S	South
SACIDS	Southern African Centre for Infectious Disease Surveillance
SADC	Southern African Development Community
SLAM	signaling lymphocyte activator molecule
SUA	Sokoine University of Agriculture
T	thymine
TADs	trans-boundary animal diseases
TAE	Tris Acetate EDTA buffer
TNRF	Tanzania Natural Resource Forum
TZS	Tanzania shilling
™	trade mark
UK	United Kingdom
UNESCO	United Nations Educational, Scientific and Cultural Organization
USA	United States of America
USD	United States Dollar
w/v	weight to volume ratio
ZVC	Zonal Veterinary Centre
µl	microlitre

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Peste des petits ruminants (PPR) is an acute, infectious, fatal viral disease of domestic and wild small ruminants such as sheep, goats, gazelles, impala and spring buck (Banyard *et al.*, 2010). The disease is transmitted by direct contact involving secretions or excretions from infected animals to healthy ones in close proximity (Muse *et al.*, 2012). It is characterized by fever, erosive stomatitis, conjunctivitis, pneumonia, gastroenteritis, mouth ulceration, diarrhea and death (Luka *et al.*, 2011; Kwiatek *et al.*, 2011). Because of its high mortality rate of up to 100% in sheep and goats, PPR is considered one of the major threats to small ruminant industry.

In Africa, PPR was first recognized as a contagious “rinderpest-like” condition in goats in Nigeria in 1930 and it was first described in Côte d’Ivoire in 1942 and later in the Benin Republic during 1944 (Lebbie *et al.*, 1994). The development of trade relations, transport, tourism and migration of wild animals susceptible to PPR contributed to the spread of the disease beyond the boundaries of western Africa (Kaukarbayevich, 2009). Recent field and laboratory data show that PPR is spreading with recent incursions in China and Bhutan and that it is moving fast towards southern and eastern Africa where it affects a wide belt of countries South of the Equator from Gabon to Tanzania and the Democratic Republic of Congo (DRC) (Libeau *et al.*, 2011; SADC, 2012).

In East Africa, antibodies against PPRV were first detected in goats in 1995 without any clinical disease and it was not until during the years 2006/07 that the disease outbreaks were reported in Kenya and Uganda, respectively (Luka *et al.*, 2011). In Tanzania, the first outbreak of PPR in sheep and goats was confirmed for the first time in December 2008 in the northern region of Arusha (Swaiet *et al.*, 2009). The outbreak is believed to have been introduced through movement of infected animals from the bordering country of Kenya. Since then, sporadic outbreaks have been reported in many parts of the country. Studies show that affected areas in Tanzania are the districts of northern, southern and eastern zones mainly Ngorongoro, Siha, Longido, Mbulu, Tandahimba, Newala and Mvomero (Swaiet *et al.*, 2009; Muse *et al.*, 2012; Kivariaet *et al.*, 2013; Lembo *et al.*, 2013; Kgotleleet *et al.*, 2014).

Phylogeny based on the viral genome reveal four lineages, I-IV, to which the PPRV may belong. Differences in genetic evolutionary characteristics of PPRV may have implications for their transmissibility, clinical manifestations in susceptible hosts and changes in the disease patterns (Banyard *et al.*, 2010, Libeau *et al.*, 2011).

Special attention has to be paid to evolutionary and epidemiological factors of PPRV from outbreaks in areas which have never been reported to have been affected. The knowledge on the genetic nature and geographic distribution of PPRV may be useful to trace the trade routes of invasion, spread and persistence of PPR and may help establish better strategies for control and eradication of the disease. This study was conducted following alleged cases of the disease in goats and sheep in Morogoro and Ngorongoro districts in Tanzania.

1.2 Problem statement and study justification

Despite the progressive efforts to prevent and control the disease, incidences of PPR still occur in small ruminants involving unaffected areas in the sub-Saharan Africa. The presence of PPR in Mvomero district and its proximity to Morogoro municipality, posed risk of spread of the disease to the later and the entire country of Tanzania (Kgotleleet *al.*, 2014).

Confirmation of PPRV infection upon occurrence of outbreaks is a compulsory and basic step prior to implementation of control and preventive measures against the disease (SADC, 2012). But, information on seroprevalence and genotype of the circulating PPRV can assist in understanding the origin, persistence, magnitude and pattern of spread of the viral strains and PPR and be applied for determining better strategic measures against the disease.

1.3 Research objectives

1.3.1 Main objective

This study was conducted to determine genetic nature of PPRV, prevalence of antibodies to the virus in goats and sheep and epidemiology of PPR in Morogoro and Ngorongoro districts in Tanzania.

1.3.2 Specific objectives

- i. To confirm PPRV infection by demonstrating the viral nucleic acid in goats and sheep suspected of PPR in Morogoro urban, Melela village and selected villages in Ngorongoro district,
- ii. To identify the lineage of the PPRV based on phylogenetic analysis of the viral nucleoprotein (N) gene,
- iii. To investigate the history of PPR introduction and causes for spread of the disease in the municipality of Morogoro and Melela village and
- iv. To determine seroprevalence of PPR in goats and sheep in selected villages within and outside Ngorongoro Conservation Area Authority (NCAA).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History and geographic distribution of PPR

PPR is a trans-boundary disease of domestic and wild small ruminants. It was first described in Côte d'Ivoire in West Africa in 1942. The existence of the disease was subsequently confirmed in Nigeria, Senegal and Ghana. By 1972 the disease had spread to Sudan (SADC, 2012). However, it is believed that the spread of PPR may not have originated from West Africa; perhaps it had existed in other places long before it was officially distinguished from rinderpest in Côte d'Ivoire (Sanz-Alvarez *et al.*, 2008 and Libeau *et al.*, 2014). It also is believed that the spread of PPR, as well as other trans-boundary animal diseases (TADs) in Africa and the Middle East, was not only related to biological factors, but also to lack of experience with regard to definitive diagnosis, surveillance and strategies for stemming the spread of the disease in the event of invasions (SADC, 2012).

Peste des petits ruminants is now endemic in the majority of Saharan and sub-Saharan African countries, Turkey, the Middle East and the Indian sub-continent. Recent isolations of PPRV have been recorded in Morocco, China, Algeria; Tajikistan, Sierra Leone, the Democratic Republic of Congo, Angola and Comoro Islands (Pope *et al.*, 2013).

2.2 Etiology of PPR

2.2.1 Classification of PPRV

Peste des petits ruminants virus is the causal agent of PPR. On account of its genetic similarity with other members of the genus *Morbillivirus*, PPRV is classified in the order *Mononegavirales*, family *Paramyxoviridae*, sub family *Paramyxovirinae*, and genus *Morbillivirus*. Morbilliviruses are highly contagious pathogens that cause some of the most devastating viral diseases of humans and animals worldwide. They include measles virus (MV), canine distemper virus (CDV), rinderpest virus (RPV), PPRV and a number of other viruses that infect aquatic mammals (Aslam *et al.*, 2009; Banyard *et al.*, 2010). Phylogenetic analysis based on sequence of the viral N gene, classifies PPRV into four lineages I-IV.

2.2.2 Morphology of PPRV

Like other members of the family *Paramyxoviridae*, PPRV is an enveloped pleomorphic particle with a lipid envelope (cell membrane-derived) with viral glycoproteins, fusion (F), and haemagglutinin (H), seen as peplomers protruding from the envelope (Fig.1). The mean diameter of PPR virion is approximately 400-500 nm (Banyard *et al.*, 2010; Munir *et al.*, 2012). The envelope encloses a genome of non-segmented, single-stranded, negative sense RNA associated with viral proteins, nucleocapsid (N) and polymerase (large) (L), forming a helical structure called ribonucleoprotein (RNP) or the core. The genome size is 15 948nt long (Munir *et al.*, 2012).

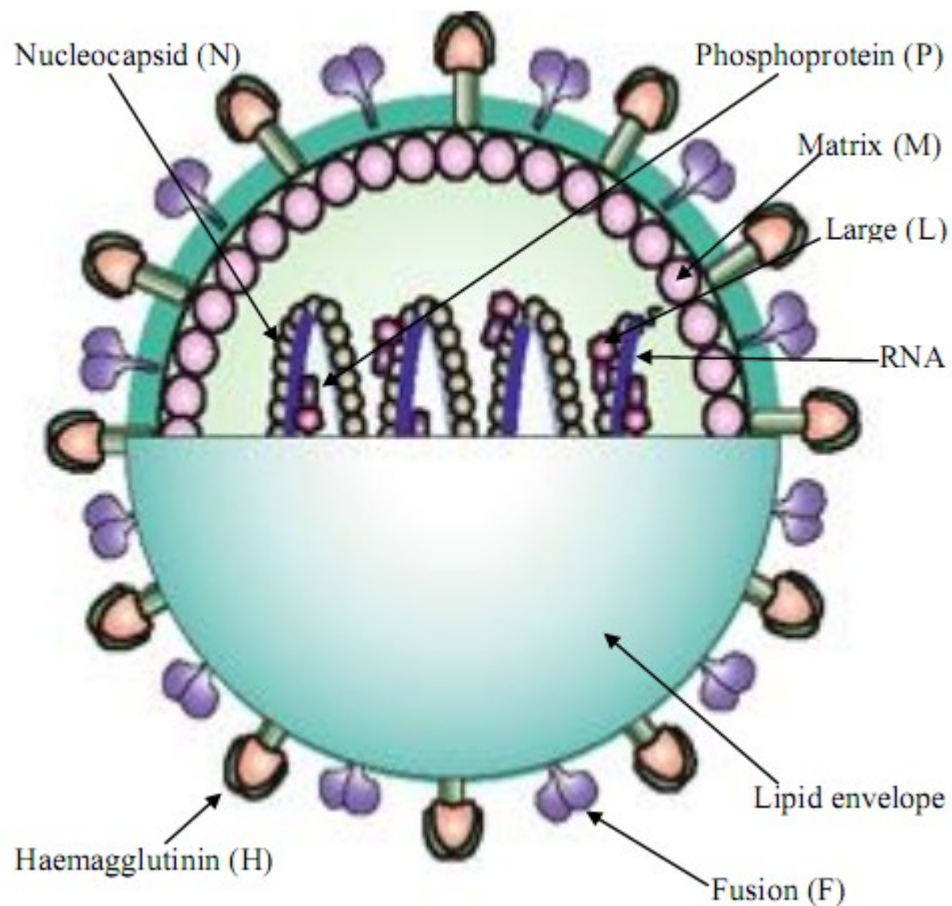


Figure 1: Schematic diagram of a PPR virus. The virus is made up of fusion (F) and haemagglutinin proteins protruding from the lipid envelope that encloses the RNA genome associated with nucleoprotein (N), large (L) polymerase and phosphoprotein (P) forming a helical structure called ribonucleoprotein or the core. Source: Munir (2015).

2.2.3 Genome organisation of PPRV

The genome encodes six structural proteins, N, P, M, F, H, L and two non-structural proteins C and V (Banyard *et al.*, 2010). The organisation of the PPRV genome is a

characteristic of morbillivirus genome; it is organised into six contiguous, non-overlapping transcription units separated by short, non-coding intergenic sequences (NCS) in the order of 3-N-P(C/V)-M-F-H-L-5 (Fig.2). The two non-structural proteins (C and V) are translated via alternative open reading frame (ORF) of the P gene and different RNA editing (Mahapatra *et al.*, 2006; Libeau *et al.*, 2014).

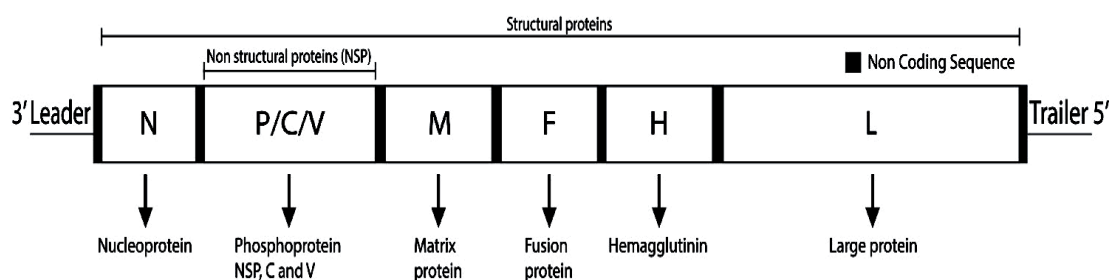


Figure 2: Genome of PPRV showing the 3-5 order of six transcription units separated by short non-coding sequences (NCS) flanked by a leader and trailer which encode for the six structural proteins nucleocapsid (N), phosphoprotein (P), matrix (M), fusion protein (F), haemagglutinin (H) and polymerase (large) protein(L). Alternative open reading frame (ORF) of the P gene produces the non-structural proteins (NSP) C and V. Source: Barrett *et al.* (1991), cited by Libeau *et al.* (2014).

2.2.4 Replication and virulence of PPRV

The major sites of viral propagation are lymphoid tissues (Pope *et al.*, 2013). Signaling lymphocyte activation molecule (SLAM) and nactin-4 on the host cell membrane serve as receptors which interact with the hemagglutinin-neuramidase

(HN) protein of PPRV. The attachment of the virus results in the fusion of the viral envelope and the host cell membrane and thus the nucleocapsid enters the host cytoplasm. The L protein then binds to the genomic promoter (GP) located just before the open reading frame (ORF) of the N gene and initiates transcription. Being a negative sense strand, the genomic RNA is transcribed into both mono- and poly-cistronic messenger RNAs (mRNAs). The produced mono-cistronic transcripts are then translated into individual viral proteins, whereby during replication the poly-cistronic mRNA becomes an antigenome which serves as a template for the production of a complete genomic RNA (Munir *et al.*, 2013; Kumar *et al.*, 2014). The mechanism of this process is not yet clear. The formed RNA is then encapsidated by the N protein.

Viral budding occurs through neuraminidase activity of the N protein, which cleaves sialic acid residues from the carbohydrate moieties of glycoproteins (Fig.3). The P, C and V proteins are expressed at various levels and perform crucial roles in facilitating the virus replication by down-regulating the host cellular interferon (IFN- α/β) responses and hence contribute to the virulence of PPRV (Munir *et al.*, 2013).

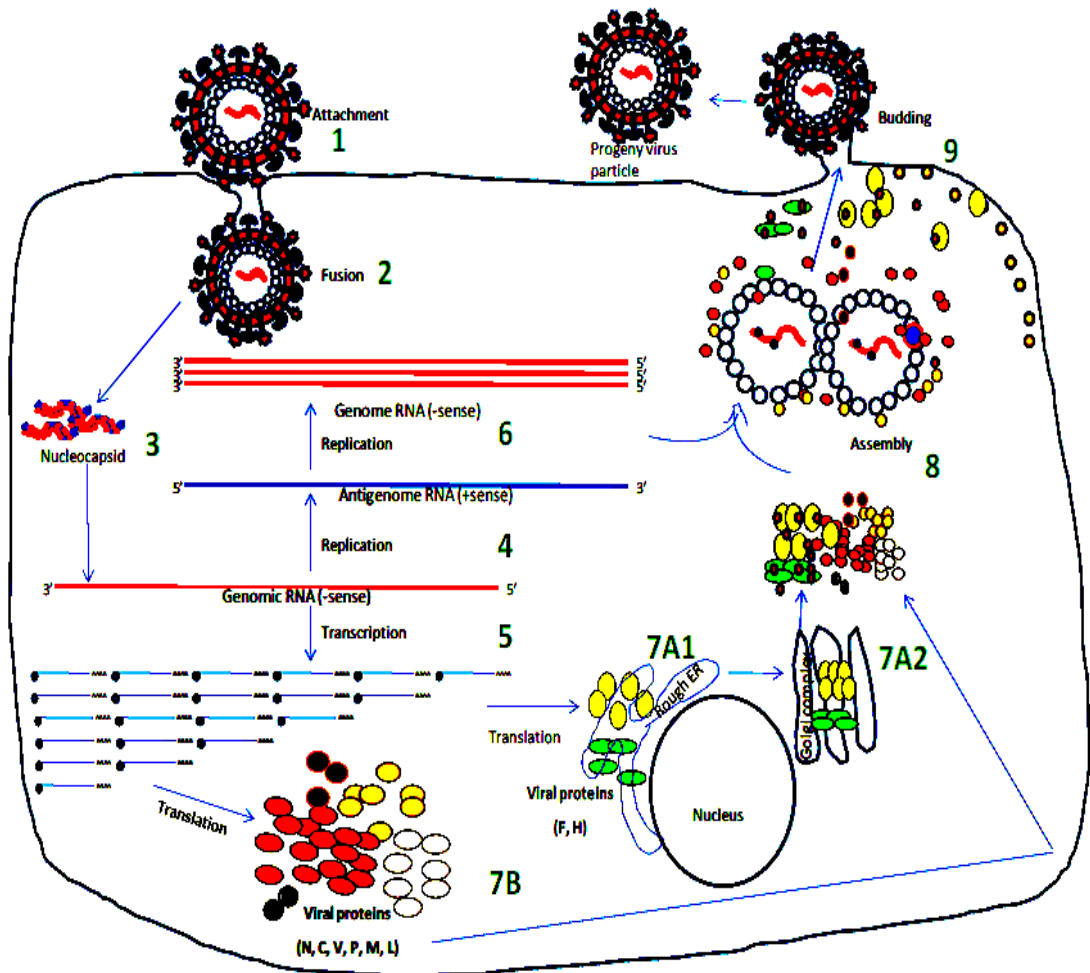


Figure 3: Replication of *peste des petits ruminants virus* (PPRV). (1). Attachment of the virus to host cell receptors (signaling lymphocyte activator molecule and nectin-4) via its HN protein. (2). Fusion with plasma membrane via the F and HN proteins (3). Release of the viral genome into cytoplasm. (4). Synthesis of full-length positive sense RNA (antigenome RNA) or complementary RNA (cRNA). (5). Messenger RNA (mRNA) synthesis by the virus-encoded RNA-dependent RNA polymerase (RdRp) in the 'start-stop' mode (a mechanism of controlling the amount of individual protein being produced). (6). Synthesis of full-length negative sense RNA

(genome RNA). (7). Synthesis of viral proteins, fusion protein (F) and haemagglutinin (H), are synthesized on rough endoplasmic reticulum (RER) (7A1) and translocated across Golgi complex (7A2), where post-translational modifications take place. Other viral proteins (nucleoprotein (N), phosphoprotein (P), matrix (M), polymerase (large) protein (L) and structural proteins (C and V), are synthesized on ribosomes (7B). (8). Assembly of progeny virions. (9). Budding of the progeny virions at the plasma membrane. Source: Kumar *et al.* (2014).

2.3 Host range and routes of transmission

Although PPR is a highly contagious disease, species such as cattle, buffaloes and pigs can be infected by PPRV but do not exhibit the clinical signs neither do they contribute to the spread of the virus (Chauhan *et al.*, 2009). Nevertheless, the report by Khalafalla *et al.*, (2010) shows that camels infected by PPRV present respiration problems, bloody diarrhoea, abortion and sudden death of apparently healthy animals.

For their ability to excrete and secrete the virus, small ruminants are regarded as the most important hosts in the transmission and spread of the virus. Movement of animals and purchase of live animals from the livestock auction markets increase the chances of interaction of potentially infected and naive animals and thus precipitate transmission and spread of the virus.

The PPRV is excreted through nasal fluid, urine, faeces, tears and saliva of infected animals. Healthy animals in close proximity with infected ones contract the virus through inhalation of infected aerosols ejected through coughing and sneezing (Kaukarbayevicch, 2009). In addition ingestion and contact with freshly contaminated materials including feed, water, floor and walls of premises play a role in the viral transmission.

2.4 Pathogenesis

The respiratory tract is believed to be the major route of PPRV infection. The findings by Pope *et al.*, (2013) suggest that after the virus penetrates the pharyngeal mucosa, it is drained to mandibular lymph nodes as well as tonsils where primary replication occurs before it enters the blood stream (viremia). Viremia starts up two to three days after infection which results in the dissemination of the virus to spleen, bone marrow, kidney, the gastro-intestinal tract (GIT) and the respiratory system (Chauhan *et al.*, 2009). Infected cells of these organs undergo necrosis leading to damage of the tissues (Fentahun and Woldie, 2012).

2.5 Symptoms and clinical signs

The typical clinical form of PPR infection is that of acute manifestations associated with discharges from the nostrils and eyes which start after an incubation period of three to six days (Fig.4). Affected animals exhibit a sudden onset of fever, severe depression, loss of appetite, clear oculonasal discharge and cutaneous nodules

(Kgotlele *et al.*, 2014). Later the nasal discharge becomes yellowish and so thicker that leads to congestion of the nostrils and hence causing respiratory distress (Chauhan *et al.*, 2009). The eyes may also become infected causing conjunctivitis with ocular discharge. Tissues in the mouth can swell and ulcers form on the lower gums, dental pad, hard palate, cheeks and tongue. Profuse diarrhoea develops in some animals due to ulceration in the mucosa of the GIT resulting in dehydration and weight loss (Luka *et al.*, 2011). Abortion may occur at any stage of gestation in infected pregnant animals (Abubakar *et al.*, 2008). The prognosis of PPR is poor and death can occur within five to ten days of the onset of fever. In acute cases the mortality rates often exceeds 70% (Sanz-Alvarez *et al.*, 2008; OIE, 2014).



Figure 4: Clinical signs of *peste des petits ruminants* (PPR). Infected goat shows mucopurulent discharges from the eyes, nostrils and mouth and cutaneous nodules. Source: Kgotlele *et al.* (2014).

2.6 Diagnosis of PPR

The nature and cause of PPR infection can be established by tentative information based on epidemiological, clinical and post-mortem investigations supported by definitive laboratory findings. The disease may be suspected when there is high fever, loss of appetite, ocular and nasal discharge, sores in the mouth and diarrhoea associated with respiratory problems and deaths in goats and sheep without involving cattle sharing the facilities (Sanz-Alvarez *et al.*, 2008; OIE, 2014). Congested nasal cavity, erosive lesions of the lining of the respiratory and alimentary tracts, soft and swollen lymph nodes are characteristic features found during post-mortem of the affected animal (Chauhan *et al.*, 2009).

Definitive diagnosis is done through laboratory tests to demonstrate PPRV infection and rule out other possible infections. Serological assays can be done for the detection of specific viral proteins or antibodies against the virus. Detection of PPRV nucleic acid is carried out using reverse transcription polymerase chain reaction (RT-PCR) using primers that target viral-specific genes. In addition, the products of RT-PCR can be sequenced and analysed for the identification of the viral lineages. Furthermore, the virus can be recovered and grown in cell lines such as Vero or Madin-Darby bovine kidney (MDBK) cells and then examined for cytopathic effect on monolayer infected with suspect material (Banyard *et al.*, 2010; Khalafalla *et al.*, 2010; Kwiatek *et al.*, 2011)

2.7 Treatment, prevention and control

Beyond palliative treatment there is no specific medication for PPR. However, there are standard measures and strategies that may be applied to control spread of the disease. Vaccination, bio-security and sanitation are the major mitigation measures against PPR outbreaks (SADC, 2012).

To maximize effectiveness of the mitigation strategies, the approach should account for prevalence and risk status of an area. Live attenuated PPRV (Nigeria 75/1 strain) can be administered (prophylaxis) across endemic and high-risk areas with livestock markets or transport routes to prevent occurrence of outbreaks (Banyard *et al.*, 2010; OIE, 2014; SADC, 2012). Quarantines and banning of livestock markets can be applied in affected or suspected areas to halt further spread of the disease.

2.8 Socio-economic impact of PPR

The adaptation of sheep and goats to different environment and production systems makes them to be the major livestock species of choice by most of the farmers including the poor. Thus, they play a significant role in the nutrition and financial income of the livestock holders and farming communities. Episodes of PPR with the mortality rates of up to 100% in sheep and goats poses a risk to about 50 million, 264 million and 1.12 billion (62.5%) domestic small ruminants in the SADC region, Africa and the world, respectively (FAO, 2012; SADC, 2012; Kumar *et al.*, 2014). This implies that the disease can result in huge losses attributable to the high morbidity and mortality. The morbidity losses such as severe weight loss, reduced

reproductive capacity and reduced milk production contribute to the fall in market value of the animals and by-products. Other economic losses are associated with the costs of treatment and vaccination and the embargo on livestock markets imposed by authorities (FAO, 2014a).

It is estimated that the total economic loss caused by PPR accounts more than 2.9 million of USD per annum during 2012-2014 (Kumar *et al.*, 2014). The incidences of PPR in Tanzania have been estimated to cause economic loss of more than 200 billion TZS (67.9 USD) per year (FAO, 2014b).

2.9 Status of PPR in Tanzania

2.9.1 Historical background

In Tanzania PPR was suspected as a ‘rinderpest-like’ syndrome in goats and sheep in the northern district of Ngorongoro in 1995 and it was officially confirmed in 2008 (Swaiet *al.*, 2009; Karimuribo *et al.*, 2011). Basing on the fact that PPR was confirmed earlier in Kenya (2007) than in Tanzania, and because the first virus reported in the country was isolated from the northern district bordering Kenya, then it is believed that the disease might have been introduced to the country through trans-boundary movement of live infected animals from Kenya (Kivaria *et al.*, 2013). This hypothesis is further supported by a sero-survey report on the absence of PPR in Tanzania by Wambura (2000) following suspected outbreak in 1998.

However, a retrospective investigation conducted in the same northern district of Ngorongoro which involved testing of retrieved samples from PPR-suspected cases showed that the disease was present in the country long before the official reports (Karimuribo *et al.*, 2011). Perhaps lack of capacity to conduct definitive diagnosis for PPR by that time contributed to the failure to recognise the disease.

2.9.2 Distribution of PPR and circulating strains of PPRV

Epidemiological studies have confirmed presence of PPR in regions of the northern, eastern, southern and central zones of Tanzania (Fig. 5). The pattern of spread of the disease is north-southwards of the country and the estimated average prevalence is 30-35% (Kivaria *et al.*, 2013). Studies show that the disease is more prevalent in regions characterised by the presence of big livestock markets or routes for nomadic livestock herds (Kivaria *et al.*, 2013; Kgotlele *et al.*, 2014).

The disease has been reported in different species including small domestic ruminants, camels and cattle (Swai *et al.*, 2011; Lembo *et al.*, 2013). However, currently there are limited reports on the presence of the disease in the wildlife of Tanzania.

Phylogenetic studies based on sequences of the N gene reveal three distinct strains of field PPR viruses circulating in Tanzania. PPRV lineage III is involved in the outbreaks in the northern and eastern regions whereby lineages II and IV in the southern regions (Kivaria *et al.*, 2013; Kgotlele *et al.*, 2014; Misinzo *et al.*, 2015).

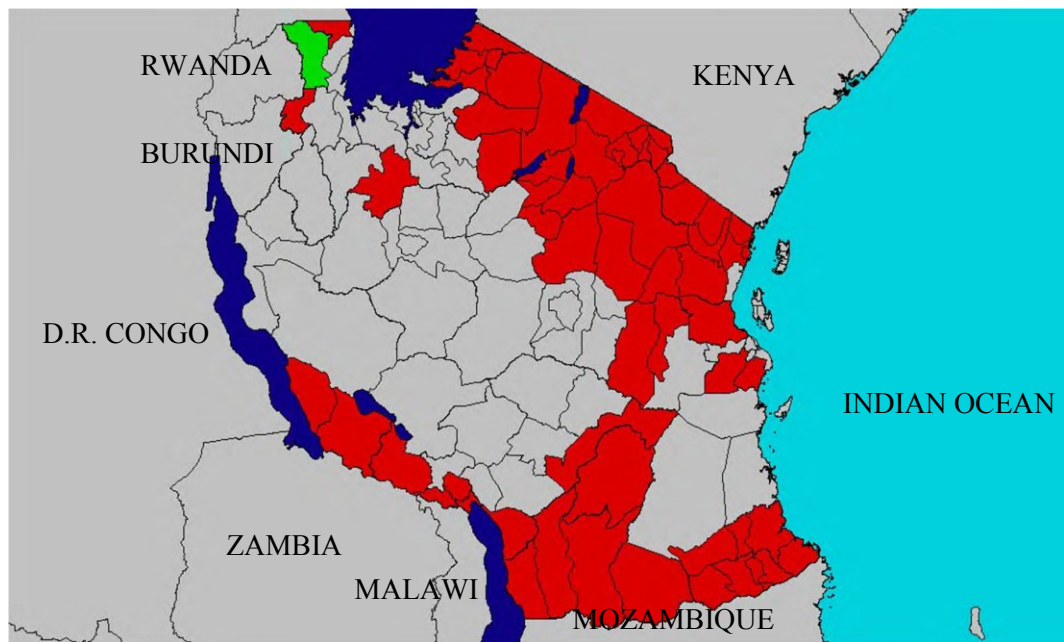


Figure 5: Spatial distribution of confirmed *peste des petits ruminants* (PPR) in Tanzania between 2008 and 2013. Affected areas are highlighted in red. Source: Malamsha (2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

This study was carried out in Morogoro and Ngorongoro districts in Tanzania following reports of suspected cases of PPR in goats and sheep.

3.1.1 Ngorongoro Conservation Area Authority

Ngorongoro Conservation Area Authority is one of the three divisions of Ngorongoro district in Arusha region in northern Tanzania. It lies at latitude $3^{\circ} 12' S$ and between longitudes $35^{\circ} 27'$ and $35^{\circ} 45' E$ with coverage of more than 59% (8292 km²) of the entire area of Ngorongoro district. The NCAA was designated in 1959 as a multiple land use area to promote biodiversity conservation and tourism where semi-nomadic pastoralists, their livestock and wildlife co-exist (NCAA, 2015; UNESCO, 2015).

The conservation area is protected and controlled by the Ngorongoro Conservation Area Authority (NCAA). For the purpose of biodiversity conservation, land cultivation has been banned as one of the strategies to control the number of people and livestock within the conservation area (Odhiambo, 2008). Hence pastoralism is the only form of land use by the inhabitants (TNRF, 2011).

Sampling for serological study was done in four villages within the conservation area namely Kakesio, Olbalba, Misigiyo and Mokilai and three villages surrounding the conservation area including Mdito, Muholo and Sukenya (Fig. 6).

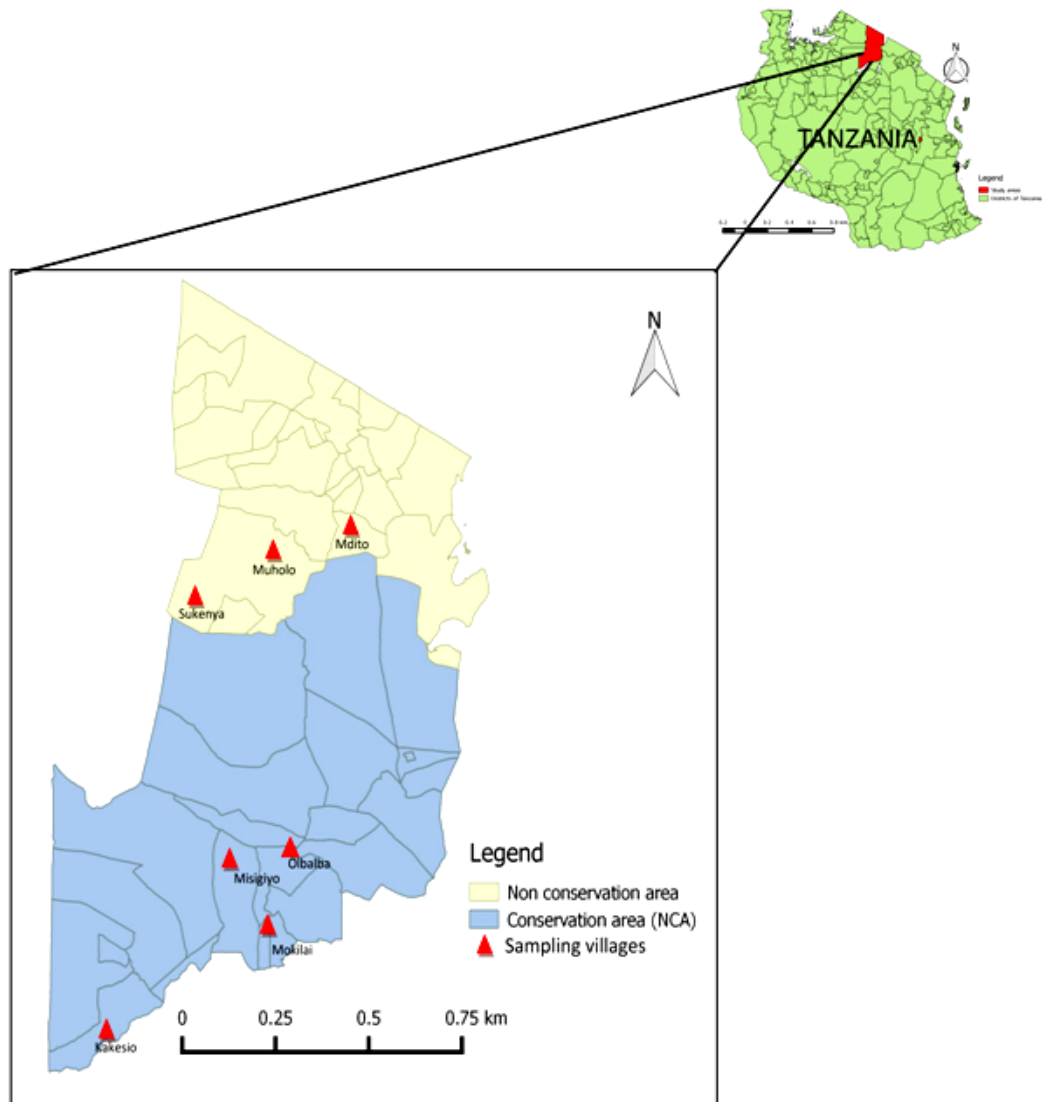


Figure 6: Sampling villages in Ngorongoro district. Conservation and non-conservation areas are highlighted in blue and grey, respectively. Sampling involved collection of caprine and ovine sera for serology.

3.1.2 Morogoro district

In Morogoro district, Morogoro urban and Melela village were involved in Molecular study of PPRV (Fig. 7). Morogoro urban is bordered to the East and South by Morogoro Rural and to the North and West by Mvomero district. It is one of the six districts in Morogoro region in eastern Tanzania. Other districts in this region include Kilosa, Kilombero, Ulanga and Gairo. It lies at latitude 4°49' S and longitudes 37°0' E. It is the regional headquarter of Morogoro. It is situated about 198km to the West of Dar es Salaam on the lower slopes of the Uluguru mountains whose peak is about 1600ft above the sea level and covers an area of 531km² (MMC, 2015). Administratively, it comprises 29 wards and forms Morogoro Municipality. There are three main rivers with several tributaries, which form a number of alluvial flood plains. These rivers are the Morogoro, Kilakala and Bigwa. Other sources of water are the Mindu Dam which was built in the late 1980s to serve for the industrial activities as well as domestic purposes (MMC, 2015). Private-owned and university animal farms located in Magadu area within the municipality (Fig. 7) were involved in the molecular and epidemiological investigation of the disease.

Melela village (Fig. 7) is located about 35 km South of Morogoro town and 67 km before Mikumi National Park by the road to southern highlands regions of Iringa and Mbeya and neighbouring countries of Malawi and Zambia. The village is a settlement for the Maasai pastoralists who came from northern Tanzania with their livestock herds in search for grazing lands. The village is famous for roasted meat

“nyamachoma” and live goats business especially for the travelers who use the main road.

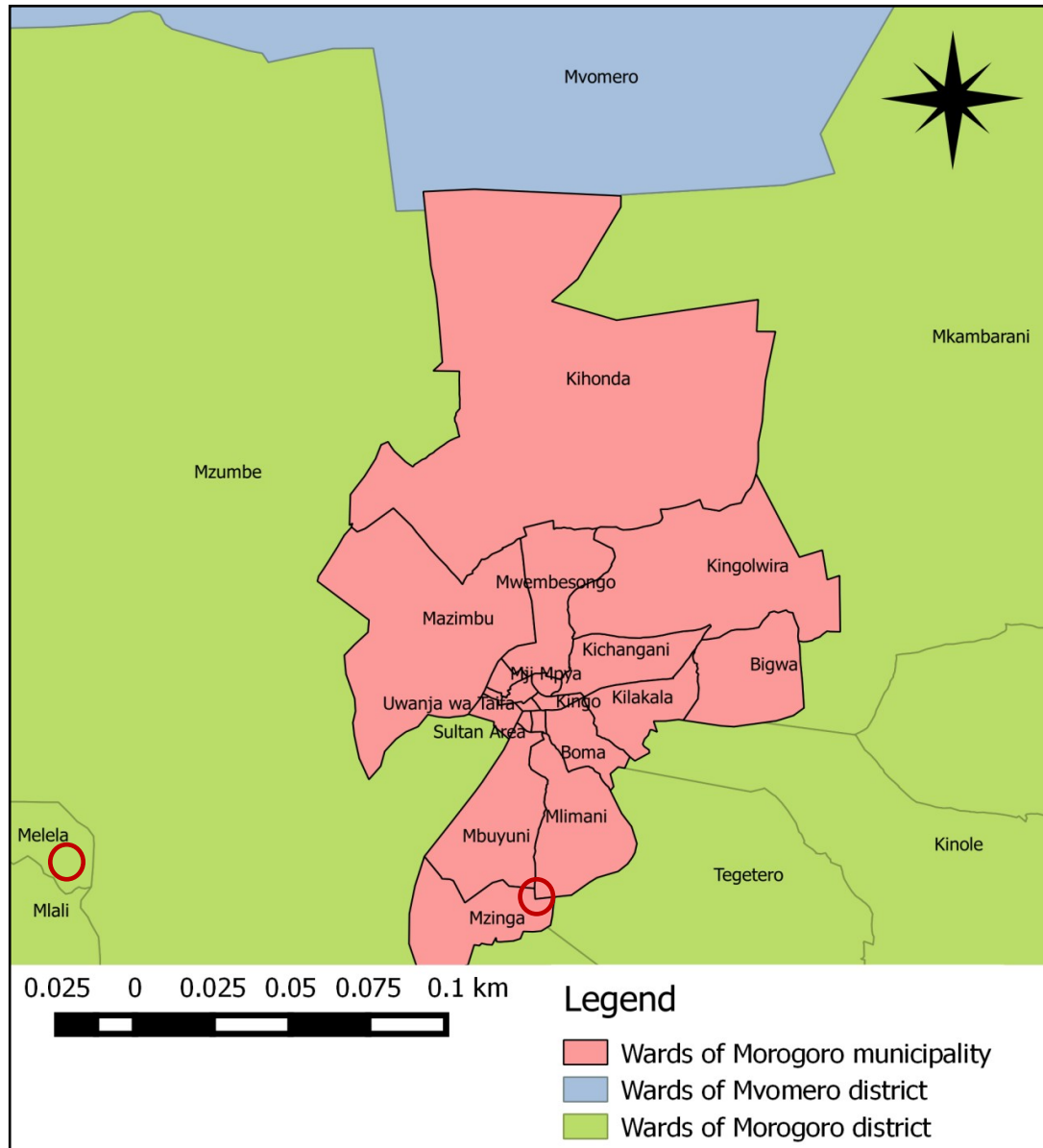


Figure 7: Study sites in Morogoro district. Red circles indicate Magadu area and Melela village where molecular and epidemiological investigation of *peste des petits ruminants* (PPR) in goats and sheep was conducted.

3.2 Study design and data collection

A purposive cross sectional study was carried out in Morogoro district for molecular and epidemiological investigation of PPRV in goats and sheep between November 2014 and January 2015 following incidences of deaths and abortions in goats and sheep in Morogoro urban and Melela village. Goats were selected purposively on the criterion of manifestation of clinical signs suggestive of PPR.

In addition, interviews were conducted to collect information about the disease and source of its introduction in the affected farms. Respondents for the interviews included personnel in charge of the ARU farm and other owners of goats and sheep who had experienced the disease in their farms.

Serum samples were retrieved from the Zonal Veterinary Centre (ZVC) Arushain October 2014. The samples were randomly collected by the ZVC staff from goats and sheep in selected villages within and outside the NCAA between April 2011 and August 2014. The study sites were selected purposively following reports of alleged cases of PPR in goats and sheep.

3.3 Sample collection and preparation

3.3.1 Pathological tissue samples

Tissue samples including mesenteric lymph nodes, lungs, spleen, liver and intestine were collected from six goats (ARU=4 and Melela=2) through postmortem of dead,

sacrificed goats and aborted goat fetuses. The tissue samples were kept in Falcon tubes and were immediately taken to the laboratory at the FVM for testing.

The tissues were homogenised separately and about 3.0ml of the homogenate was suspended in 3.0ml of phosphate-buffered saline (PBS, pH 7.2). After a thorough stirring, the suspension was decanted and centrifuged for 10 minutes at $1500 \times g$ for five minutes to obtain cell-free supernatant. About 1.0ml of each supernatant was decanted into sterile microcentrifuge tubes and stored at -40°C until RNA extraction.

3.3.2 Serum samples

A total of 252 serum samples were collected by jugular vein puncturing from goats and sheep in selected villages within and outside NCAA. The sera were shipped in a cool box with ice packs to SUA Morogoro and upon arrival they were stored at -40°C until testing.

3.4 Sample analysis

3.4.1 Serological analysis

A standard competitive enzyme-linked immunosorbent assay (cELISA) technique was employed for the detection of antibodies to PPR virus in serum samples by using Biological Diagnostic Supplies Limited (BDSL, UK) kit (Anderson *et al.*, 1991). The test is based on competitive inhibition between the mouse anti-PPR monoclonal

antibody (MAb) and antibodies in the test serum binding to the PPR antigen coated onto the wells of a micro-well plate. The presence of the antibodies to PPRV in test serum results in a reduction in the expected colour intensity following the addition of horseradish peroxidase (HRP)-conjugated rabbit antimouse antibody and substrate solution (hydrogen peroxide with ortho-phenylenediamine).

The assays were carried out using flat-bottomed, 96 well micro-well plates (NUNC, Denmark) according to the procedure as described in the manual by the manufacturer. However, satisfactory results were not obtained using 1:100 dilutions of the reconstituted PPR antigen as per instructions. Therefore, 1:50 dilutions of the antigen were used and 20 μ l instead of 10 μ l volumes of test and control sera were added to the appropriate wells as illustrated in Appendix 1. The micro-well plates were read on ELISA reader (Erba LisaScan IITM, Mannheim, Germany) to give optical density (OD) of each well at a wavelength of 492 nm.

The obtained OD values were used for calculating percentage inhibition (PI) values of the control and test sera by using the formula $PI \text{ of serum} = 100 - (\text{average OD of serum} / \text{OD of control serum}) \times 100$. The reliability of the assay performance was decided based on the OD values for the monoclonal antibody control (C_m), and PI values for the positive control (C⁺), negative control (C⁻) and conjugate control (C_c). All acceptance criteria were according to the manufacturer's instructions, whereby the upper and lower limits of OD values for the C⁺ had to read 1.3 and 0.3 respectively, the PI value for the C⁺ at least 50%, the PI value for the C⁻ not less

than 25% and for the Cc the PI value had to range from 95% to 105%. In addition, the PI values for the Cm had to be at least 50%.

The test sera were replicated on the same micro-well plate. For a test serum to be considered as positive, each of the two replicates had to produce a PI value of at least 50%, whereas test sera giving mean PI values less than 50% were regarded as negative.

3.4.2 Molecular analysis

3.4.2.1 Extraction of viral RNA

Viral RNA was purified from the tissue homogenates, 20 sera which gave higher PI values for antibodies to PPRV and vaccine (Nigeria 75/1) using QIAamp Viral RNA Mini Kit (QIAGEN® Hilden, Germany). The kit combines the selective binding properties of a silica gel-based membrane with the centrifugation speed to isolate intact RNA free of protein, nucleases, and other contaminants and inhibitors.

Extraction was carried out according to the manufacturer's instructions. The procedure involved lysing the samples under highly denaturing conditions by using a lysing buffer to inactivate RNases and to ensure isolation of intact viral RNA. The lysed samples were then loaded onto the QIAamp Mini columns (supplied with the kit) to allow viral RNA to bind to the membrane. Contaminants were washed away in two steps using two different wash buffers of the kit and centrifugation at 6000 ×g for one minute. The RNA bound to the membrane was eluted in a special RNase-free

buffer and collected into sterile 1.5ml microcentrifuge tubes and stored at -20°C until analysis.

3.4.2.2 Reverse transcription and amplification of viral nucleic acid

Analysis of PPR virus RNA was carried on Veriti thermocycler machine (Applied Biosystems, USA) by using SuperScript® III One-Step RT-PCR System with reverse transcriptase and Platinum® Taq DNA Polymerase (Invitrogen, Calsband, CA). The formulation of this kit allows reverse transcription of RNA and subsequent amplification of the resulting complimentary DNA (cDNA) by polymerase chain reaction (PCR) to take place in a single tube. The amplification of cDNA was achieved by targeting a region of PPRV N gene using NP3 (5-TCT CGG AAA TCG CCT CAC AGA CTG-3) and NP4 (5-CCT CCT CCT GGT CCT CCA GAA TCT-3) primers (Couacy-Hymann *et al.*, 2002).

Analysis procedures were carried out according to the manufacturer's instructions. The samples were analyzed along with PPRV vaccine extract and RNA-free water to serve as positive and negative controls, respectively.

3.4.2.3 Resolution and visualization of PCR products

Five microliters of the PCR products were resolved along with 1.0µl of a 1000bp DNA size marker, GeneRuler, (Thermo Scientific, Calsband, USA) by electrophoresis on a 1.5% w/v agarose gel containing 2µl of GelRed nucleic acid stain (Phenix Research Products, USA). The electrophoresis was carried out using

0.5× TAE buffer at 100V for 30 minutes. Resolved PCR products were visualized and recorded by using a Gel Doc™ EZ gel imaging system (Bio-Rad, California, USA).

3.4.2.4 Sequencing of PCR amplicons

PCR products were purified by using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and sequenced directly by using Big Dye Terminator version 3.1 sequencing kit (Applied Biosystem, Foster City, CA, USA) following the manufacturer's instructions. Sequenced PCR products were separated using an ABI 3500 Genetic Analyser (Applied Biosystem, Foster City, CA).

3.5 Data analysis

3.5.1 Phylogenetic analysis

Editing and assemblage of the nucleotide sequences generated from sequencing reaction of the RT-PCR products of the positive samples was done by using Sequence Scanner V1.0 (Applied Biosystem, Foster City, CA, USA). Nucleotide sequences were manually edited using Microsoft Notepad to create a consensus DNA sequence. Homologous nucleotide sequences were searched and retrieved from the nucleotide sequence database, GenBank of the NCBI, using BLAST®. Alignment of the resultant nucleotide sequence with those retrieved from the database was performed in MEGA 6 programme (CEMI, Tempe, AZ, USA) by employing CrustalW algorithm. Phylogenetic tree was constructed by employing Neighbour-

joining method and bootstrap was used for checking the validity of the phylogeny based on 1000 replications.

3.5.2 Statistical analysis

Data were listed, selected, sorted and manipulated using Microsoft Excel sheet. A statistical tool, Epi Info 7, was used to calculate proportions of animals positive to PPR and to test significance of the associations between PPR seropositivity and risk factors including study areas and species of the animals at $P < 0.05$ by employing Chi-square test.

CHAPTER FOUR

4.0 RESULTS

4.1 Clinical signs and epidemiological findings

Animal Research Unit farm was visited for the purpose of investigating PPR in mid January 2015 following reports of presence of flu-like symptoms, diarrhoea, deaths and abortions in goats. During the visit clinical signs including depression, thick and yellowish discharges from the eyes, nostrils and mouth, respiratory problem and diarrhoea were observed in four goats (Fig.8). Five deaths and eight abortions were also reported to have occurred among the goats. However, sheep, pigs, cattle and camels at the farm were not affected. The outbreak also affected herds of neighbouring farms in Morogoro urban and extended to Melela village located about 35 km from Morogoro town where the viral infection was confirmed in two goats following report of alleged outbreak which persisted for two months from December 2014 to January 2015.



Figure 8: Clinical features observed in goats with *peste des petits ruminants* (PPR) in Magadu and Melela village. Goats were seen with mucopurulent nasal (A and B) and ocular (C) discharges. Mortality (D) was also observed among goats with clinical signs reminiscent of PPR.

4.2 Postmortem findings

Pneumonia and purple-reddish areas which are firm to touch on the lungs and froth-filled trachea in goats suspected of PPR were observed during post mortem investigation (Fig. 9).

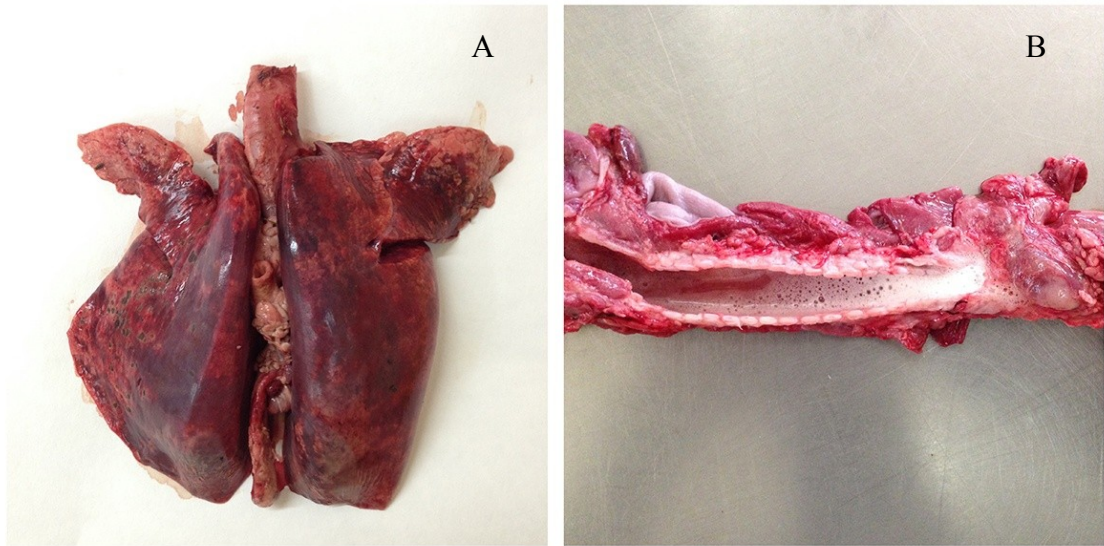


Figure 9: Post mortem findings in organs of goats that died from *peste des petits ruminants* (PPR) showing lungs with pneumonia featured with purple-reddish areas which are firm to touch (A) and froth-filled trachea (B).

4.3 Confirmation of PPRV infection

Peste des petits ruminants virus was detected in spleen and liver of two goats (one from ARU in Magadu area and the other from Melela village) through the amplification of the viral N gene by one-step RT-PCR. Amplicons with an expected size of 351bp were obtained (Fig. 10). None of the 20 selected serum samples from Ngorongoro tested positive for PPRV nucleic acid despite of their high PI values for antibodies against the virus.

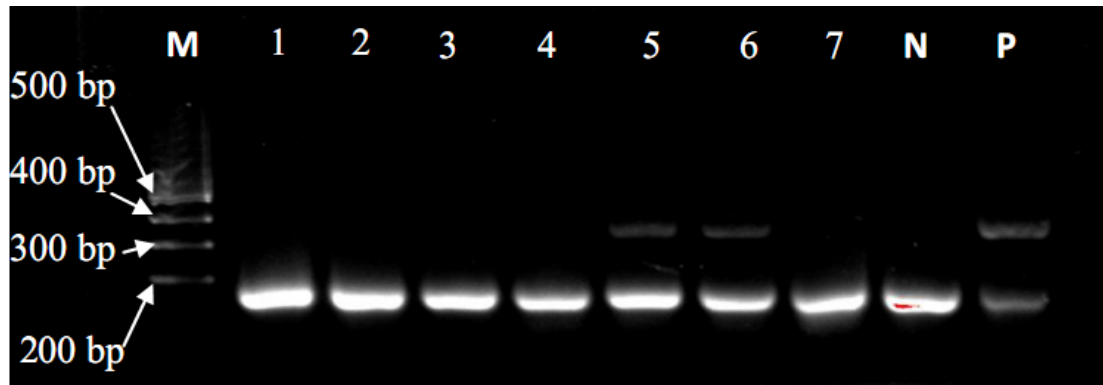


Figure 10: Detection of *peste des petits ruminants virus* (PPRV) using reverse transcription polymerase chain reaction (RT-PCR) followed by electrophoresis in agarose gel. Lane **M** is DNA size marker, 1-7 are test samples whereby **N** and **P** are negative and positive controls, respectively. Samples 5 and 6 are positive for the 351 bp nucleoprotein (N) gene of PPRV.

4.4 Phylogeny of PPR virus

Two consensus sequences of 351 nucleotides for the viral N gene were yielded from the positive samples. The two sequences were 100% identical. Phylogenetic analysis showed that the PPR virus involved in the outbreak in Morogoro district belongs to the lineage III (Fig. 11) and was closely related to the Tanzanian strains reported previously by Kgotlele *et al.*, (2014) in Dakawa and Ngorongoro with nucleotide sequence identity of 99% and 98%, respectively (Table 1). The alignment of the sequences revealed nucleotide substitutions in two homologous positions. Two cytosine (C) nucleotides in the Dakawa isolate from Mvomero district were replaced by adenine (A) and thymine (T) in those of Magadu or Melela (C→A and C→T). One of these substitutions occurred within the NP4 primer sequence. The obtained

PPRV nucleotide sequences from Magadu and Melela were submitted to the GenBank database and were assigned accession numbers KT989870 and KT989871, respectively.

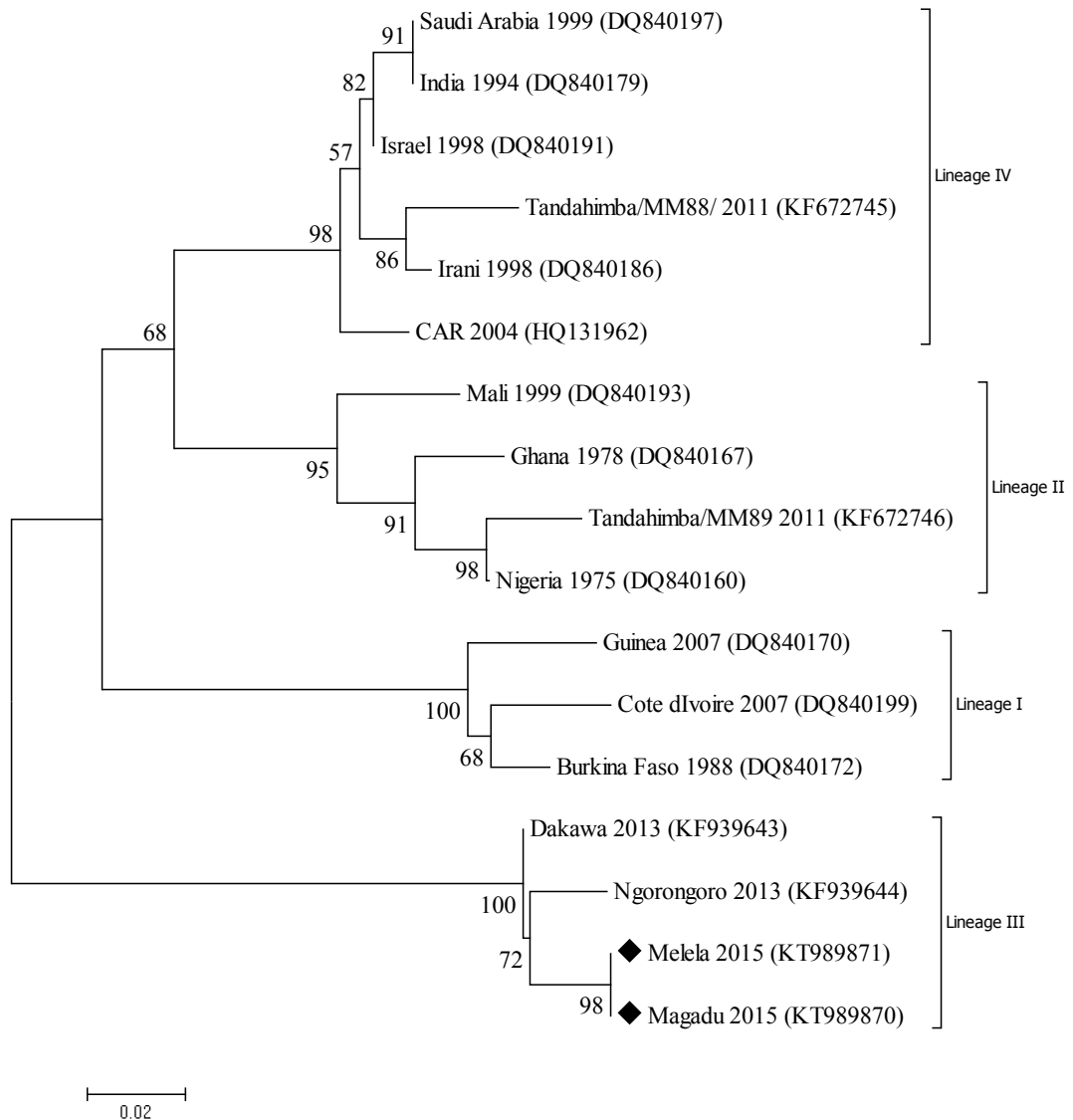


Figure 11: Phylogenetic analysis of *peste des petits ruminants* virus (PPRV) based on the viral nucleocapsid (N) gene nucleotide sequences obtained from this study (marked with black diamond) and ones retrieved from the GenBank database representing the four lineages I-IV. The analysis was done using Neighbour-joining algorithm of the Molecular Evolutionary

Genetics Analysis (MEGA). The phylogeny was tested based on 1000 bootstrap replications and the validity values are included in the branches of the tree.

Table 1: Comparison of the *peste des petits ruminants* virus (PPRV) detected in this study with others reported earlier based on the nucleocapsid (N) gene nucleotide sequences retrieved from the GenBank

Sequence name	Country	Year	Accession number	Lineage	Identity (%)
Dakawa	Tanzania	2013	KF939643	III	99
Ngorongoro	Tanzania	2013	KF939644	III	98
Accra/78	Ghana	1978	DQ840167	II	83
CAR04	CAR	2004	HQ131962	IV	83
Isolate 75/1	Nigeria	1975	DQ840160	II	83
Isolate 98/10	Israel	1998	DQ840191	IV	83
Isolate 98/3	Iran	1998	DQ840186	IV	83
Isolate 99/8	Saudi Arabia	1999	DQ840197	IV	83
Sassayan 94/2	India	1994	DQ840179	IV	83
Isolate 99/366	Mali	1999	DQ840193	II	82
Isolate 88	Guinea	2007	DQ840170	I	81
Goat/Tandahimba /MM88/Tanzania/ 2011	Tanzania	2011	KF672745	IV	81
Goat/Tandahimba /MM89/Tanzania/ 2011	Tanzania	2011	KF672746	II	81
Isolate 88	Burkina Faso	1988	DQ840172	I	81
Isolate 89	Cote d'Ivoire	2007	DQ840199	I	80

4.5 Introduction and spread of PPR in Morogorourban

Animal Research Unit harbours different species of livestock including 70 goats, 31 sheep, 44 cattle, 4 camels and 28 pigs reared in semi-intensive system. While the

goats and sheep are enclosed in different chambers under the same roof, cattle, camels and pigs are enclosed separately. The ruminant animals share the same pasture at Miembemiwili area, located about one kilometer from the farm.

The interviews conducted in this study showed that the “flu-like” symptoms accompanied by depression, diarrhoea, abortions and deaths started to appear in goats a few days after introduction to the farm of 38 purchased goats from Mkongeni livestock auction market in Mvomero district in November 2014. The goats were apparently healthy when they were purchased and a veterinary officer at the market confirmed vaccination of the animals against PPR. Vaccination status of the resident animals at the farm prior to the introduction of the purchased ones was not known by the respondents.

It also was revealed that private-owned herds around ARU were affected even more severely. Thirteen out of 47 goats died and more than eight cases of abortion occurred in one flock between December 2014 and January 2015. The respondents described the disease by comparing it with the one that severely affected goats and sheep previously in Ngurundege village in Mvomero district. However, the respondents had no history of introduction of new animals to their herds that could have been the source of the outbreak, but their herds frequently contact herds from other farms including ARU during grazing at Miembemiwili.

4.6 Seroprevalence of PPR in selected villages of Ngorongoro

A total of 252 tested serum samples were collected from four villages within NCAA (n=140) and three villages bordering NCAA (n=112). The samples were categorized in terms of species as 71.03% goats and 28.97% sheep. Overall results of cELISA showed that 58.33% (CI: 51.98%-64.49%) of the animals were positive for antibodies to PPR virus (Table 2).

Prevalence of antibodies to PPRV in the animals was significantly associated with the sampling area ($P=0.0004$), whereby higher percentage of seropositive animals (70.54%, CI:61.18%-78.77%) was from villages outside the conservation area than from within (48.57%, CI: 40.04%-57.16%) (Fig. 12). The highest percentage of seropositive animals was from Mdito village (86.36%) and the lowest percentage was from Olbalba village (28.56%) located outside and within the conservation area, respectively.

Table 3 shows that there was a significant difference in the distribution of antibodies to PPRV with regard to species of the animals, whereby percentage of seropositive goats (63.69%, CI: 56.18%-70.73%) was higher than that of sheep (45.21%, CI: 33.52-57.30%) ($P=0.0069$).

Table 2: Overall seroprevalence of *peste des petits ruminants* (PPR) in selected villages within and outside the conservation area

Location	Tested samples	Positive	Prevalence (%)	95% Confidence limit
Within NCAA	140	68	48.57	40.04-57.16
Outside NCAA	112	79	70.54	61.18-78.77
Total	252	147	58.33	51.98-64.49

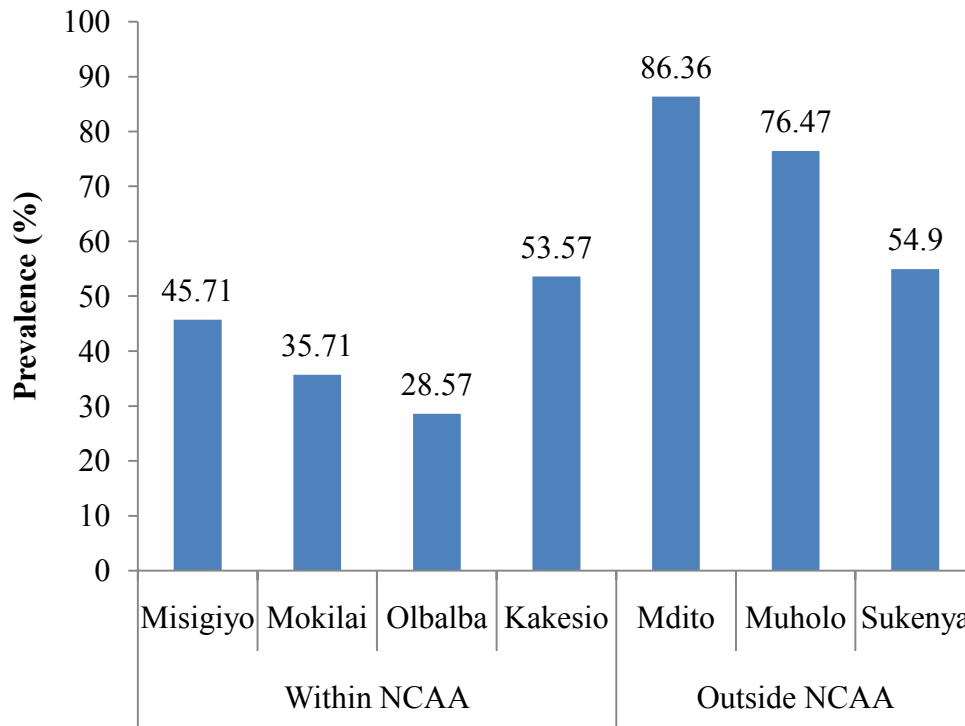


Figure 12: Distribution of antibodies against *peste des petits ruminants* virus (PPRV) in goats and sheep from selected villages within and outside Ngorongoro Conservation Area Authority (NCAA). Higher seroprevalence was found in villages located outside NCAA with highest prevalence in Mdito village while the lowest seroprevalence was found in Olbalba village located in the conservation area.

Table 3: Distribution of antibodies *topeste des petits ruminants virus* (PPRV) in selected villages of Ngorongoro district based on species of the tested animals

Village	Caprine			Ovine		
	Tested samples	Positive	Prevalence (%)	Tested samples	Positive	Prevalence (%)
Misigiyo	2	1	50.00	33	15	45.45
Mokilai	0	0	0	14	5	35.71
Olbalba	7	2	28.57	0	0	0
Kakesio	84	45	53.57	0	0	0
Mdito	43	38	88.37	1	0	0
Muholo	9	8	88.89	8	5	62.50
Sukenya	34	20	58.82	17	8	47.06
Total	179	114	63.69	73	33	45.21

CHAPTER FIVE

5.0 DISCUSSION

Peste des petits ruminants is a trans-boundary disease of small ruminants which is mainly distributed through movement of infected animals to naive areas. Tanzania is among the countries affected by the disease. The findings of this study confirm field PPRV infection in suspected goats within Morogoro district and presence of antibodies against the virus in sheep and goats in Ngorongoro district.

Information gathered through the interviews suggests that the recent introduction of the purchased goats from Mkongeni livestock market located in the affected district of Mvomero could be the cause of the present PPR outbreak in Morogoro urban. This is supported by the finding of the phylogenetic analysis which shows that the PPRV detected in the current outbreak belongs to lineage III similar to that reported previously by Kgotlele *et al.* (2014) in the outbreak that occurred in Dakawa in Mvomero district and the two were 99% identical. These observations are comparable with the findings by Muse *et al.* (2012) who found that purchased goats from a livestock market in Dar es Salaam were the cause of PPR introduction in Tandahimba and Newala districts in southern Tanzania. These findings support the hypothesis that trading of small ruminants is the most likely reason for the spread of PPR (Sanz-Alvarez *et al.*, 2008).

Observation of nucleotide substitutions in two homologous positions of the vial N gene nucleotide sequences between the Dakawa isolate and the ones found in this

study contradicts the finding by Sevik and Sait (2015) who reported that PPRV does not undergo rapid genetic changes in the N gene.

In addition, this investigation reveals the source of spread of the disease in Morogoro urban to be the interaction of healthy and infected herds in a common grazing area called Miembemiwili located within the municipality. Although the outbreak extended to Melela village, there is no evidence of the specific source of exposure of goats and sheep to PPR in the village. But, it is convincing to suggest that marketing of live goats and sheep and interaction of livestock herds in the village with potentially infected herds from neighbouring villages in the affected district of Mvomero as a result of movement of herds in search for forage and water practiced by the Maasai pastoralists, might be the source of PPR introduction into the village.

The major mitigation measure employed in the country against the disease is vaccination. The seroprevalence results of more than 58% found in the present study in selected villages of Ngorongoro district shows that despite the efforts that have been made against PPR since the official report of outbreak in the district in 2008, the disease is still persisting. The persistence of the disease in the areas affected by PPR could be attributable to low coverage of immunization of small ruminants against PPRV infection or unrestricted movement of animals. This is supported by the observation that the villages within the conservation area into which introduction of livestock herds from other areas is restricted by the authority, NCAA, had low prevalence of PPR as compared with those outside the conservation area where there is freedom of livestock movement. This observation suggests that restriction of

livestock introduction into the conservation area does not only help control the number of livestock for the purpose of biodiversity conservation, but also limits the chances of contact between resident animals and ones from other areas, hence reducing the potential for transmission of animal diseases into the area. In addition, lack of vaccination history in some villages outside the conservation area may have contributed to the high percentage of the overall prevalence of PPR in Ngorongoro district. Also, the difference in vaccination history between the two areas may account for the observed high seroprevalence in the villages outside the NCAA as being caused by field viral infection, whereas the low antibodies to PPRV in the animals from the conservation areas due to vaccination. The failure to demonstrate the PPRV nucleic acid in the caprine and ovine sera samples from Ngorongoro despite the high PI values for antibodies to the virus in this study, suggests that the animals were previously infected by PPRV and that they survived the infection.

In this study, the percentage of goats with PPRV antibodies was found to be significantly higher than that of sheep. This observation is consistent with those noted by Abubakar *et al.* (2008) and Farougou *et al.* (2013). However, from this observation alone it is difficult to argue that sheep have a better chance to PPRV infection than goats since other similar studies found higher seroprevalence in sheep than in goats (Özkul *et al.*, 2002 and Munir *et al.*, 2008). Absence of information on the clinical status of the tested animals in this study limits the comparison of the impact of the infection among the animals. The varying trend of seroprevalence results between the two species could be attributable to sampling error.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Since its confirmation in Ngorongoro district in 2008, the efforts to contain PPR in Tanzania have not been a good success. With the seroprevalence result of more than 58% in the selected villages of Ngorongoro and the identification of the field strain (lineage III) of PPRV involved in the recent outbreak in Morogoro urban, the present study provides evidence that PPR is not only persistent in previously affected areas but also is actively spreading to naive areas of the country due to trading of live goats and sheep and that the virus undergoes rapid changes in the N gene.

6.2 Recommendations

The findings of this study show that PPR was introduced into Morogoro urban through purchased goats from a livestock market in the affected district of Mvomero. In order to contain the disease, it is recommended that movement of livestock from endemic areas should be restricted until the absence of the disease is guaranteed. To achieve this, the veterinary authorities should be dedicated and vigilant like the NCAA. In addition, further studies should be carried out to assess the effectiveness of the control measures taken so far in the event of outbreaks and find alternatives if the current ones are found ineffective.

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APPENDICES

Appendix 1: A sketch of ELISA plate showing layout of positions for controls and test sera.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cc	Cc	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
B	Cc	Cc	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
C	POS	POS	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
D	POS	POS	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
E	NEG	NEG	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30
F	NEG	NEG	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30
G	Cm	Cm	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40
H	Cm	Cm	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40