Establishment of reverse genetics system for PPR virus to develop recombinant vaccines

A thesis submitted for the degree of Doctor of Philosophy

The University of Warwick

School of Life Sciences

and

The Pirbright Institute

By

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2015

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Acknowledgements

First and foremost, I would like to thank Professor Satya Parida for provided expert guidance and supervision during my PhD research. I would like to thank co-supervisors Dr. Ashley C Banyard and Dr. Carrie Batten for their advice and support. I would like to thank university supervisor Professor Andrew J Easton for assistance at The University of Warwick.

I am grateful to the past and present members I have worked in Vaccine differentiation group at The Pirbright Institute. A special thanks to Dr Aravindh Babu and Dr Mana Mahapatra for their expertise and advice. I must also thank other members of the group Anuj Tewari, Krupali Parekh, Sasmita Upadhyaya, and Katie Lloyd-jones for their general help and assistance.

I would like to thank Biotechnology and Biological Sciences Research Council, UK and Department for International Development, UK for funding my project and studentship (Combating infectious diseases of livestock for international development grant, number BB/H009485/1).

Finally, most of all I want to thank my mum, dad and brother for everything and without their love, patience and understanding I wouldn't have made it this far.

Declarations

The work in this thesis has not been submitted at any time for examination for another degree. Some of the work presented in the thesis has been published in peer reviewed journals. I further declare below in detail all contributions made by others towards the project and the final written manuscript.

Chapter 1 General introduction

All the text and figures were made by Murali Muniraju except two figures that were reproduce from the scientific literature with the appropriate citation in the figure legends.

Chapter 2 Materials and methods

The text has been written and presented in full by Murali Muniraju except one figure that were reproduce from the scientific literature with the appropriate citation in the figure legends.

Chapter 3 PPRV complete genome sequencing and analysis

All the sequencing work and analyses have been performed by Murali Muniraju. The draft chapter has been prepared by Murali Muniraju. The drafts of the publication that resulted from this work and the thesis chapter have been improved following input from my supervisors Prof Satya Parida, Dr Carrie Batten and Dr Ashley Banyard. The revised version has been checked by Prof Satya Parida, Dr Ashley Banyard and the University supervisor, Prof Andrew Easton.

The complete genome sequence for the PPRV Ethiopia 1994 isolate was generated and supplied by Dr Muhammad Munir and Dr Genevieve Libeau. Drs Gelagay Ayelet and Mana Mahapatra supplied the Ethiopia 2010 Lineage IV virus whilst Dr Chrisostom Ayebazibwe provided the Uganda 2012 lineage III PPRV isolate. Drs Mehdi El Harrak and Aravindh Babu R. Parthiban provided the Morocco 2008 lineage IV virus isolate. Drs Jingyue Bao and Zhiliang Wang provided the RACE protocol and trained Murali Muniraju to enable generation of sequence for the

genome and anti-genome promoter regions. Dr Vincent Michaud (CIRAD, Montpellier, France) was involved in the critical reading of the manuscript.

Chapter 4 Establishment of reverse genetics system to rescue the Nigeria 75/1 PPR vaccine strain for the development of marker vaccines

All the lab work was carried out by Murali Muniraju. Drafts for both the publication and chapter have been written by Murali Muniraju. As with chapter 3, comments were obtained from Prof Satya Parida, Drs Ashley Banyard, Carrie Batten and Mana Mahapatra and the draft was improved accordingly. Dr Hubert Buczkowski had performed the phage display to determine the proposed C77 binding regions during his PhD study. The revised thesis chapter was read and commented on by Prof Satya Parida, Dr Ashley Banyard and Prof Andrew Easton.

Chapter 5 *In vivo* evaluation of the rPPRV-C77c Nigeria 75/1 rescued vaccine virus and its comparison with the parent PPRV Nigeria 75/1 vaccine strain

The writing and all lab work was carried out by Murali Muniraju. The draft was been improved by addressing the comments from Prof Satya Parida, Drs Carrie Batten and Dr Ashley Banyard. Dr Mana Mahapatra assisted Murali Muniraju with virus isolation from experimental samples. Prof Satya Parida conducted the animal experiment within the isolation unit. A revised thesis chapter was read and commented on by Prof Satya Parida, Dr Ashley Banyard and Prof Andrew Easton.

Chapter 6 Segmentation of PPRV to generate multivalent vaccines

All the lab work and writing was performed by Murali Muniraju. All written work was commented on by Prof Satya Parida and Dr Ashley Banyard. The revised thesis chapter was read and commented on by Prof Satya Parida, Dr Ashley Banyard and Prof Andrew Easton.

Chapter 7 General discussion and further perspectives

The chapter was written by Murali Muniraju and improved upon following comments from Prof Satya Parida and Dr Ashley Banyard. The revised thesis chapter was read and commented on by Prof Satya Parida, Dr Ashley Banyard and Prof Andrew Easton.

Summary

Across the developing world peste des petits ruminants virus (PPRV) places a huge disease burden on small ruminant agriculture. PPR is mainly controlled by vaccinating animals with live attenuated vaccines. However, the current PPR vaccines and companion serological tests do not enable serological differentiation between naturally infected and vaccinated animals (DIVA), therefore a meaningful serological assessment of vaccine coverage and epidemiological surveillance is not possible. Therefore, the main objective of this PhD study was to establish a reverse genetics system for PPRV, so that a marker vaccine could be developed to enable the serological differentiation between vaccination and infection, alongside developing proof of concept for increasing the valency of the existing vaccines.

Initially, as a prerequisite to full genome synthesis the full genome sequence for a PPRV vaccine strain was confirmed. An efficient reverse genetics system for the PPRV Nigeria75/1 vaccine strain was established in this study and 3 recombinant PPRVs were rescued including a faithful clone of the vaccine strain (rPPRV Nigeria75/1), a clone expressing GFP as a heterologous protein (rPPRV+GFP Nigeria75/1) and a negatively marked vaccine containing mutations to the haemagglutinin (H) gene (rPPRV-C77 Nigeria75/1). All 3 rescued viruses showed similar growth characteristics *in vitro* when compared to the parental vaccine strain and, following *in vivo* assessment the H mutant provided full protection in goats upon virulent virus challenge. Although the mutations made to H abrogated *in vitro* binding of C77, the mutations made were not sufficient to enable DIVA *in vivo*.

Finally proof of concept was developed for the segmentation of PPRV and expression of heterologous proteins in an effort to generate a multivalent vaccine. A recombinant two-segmented version of PPRV was successfully rescued that expressed GFP from one segment and the bluetongue virus VP2 from the other. This virus was partially characterised *in vitro* and demonstrates the potential for this approach in the development of multivalent vaccines for small ruminants.

List of abbreviations

3D	3-dimensional (3D)
DAPI	4’,6 diamidino-2-phenylindole
$TCID_{50}$	50% tissue culture infectious dose
aa	Amino acids
A	Alanine
AGP	anti-genome promoter
BF	Bayes factor
BEAST	Bayesian evolutionary analysis sampling trees
BSP	Bayesian skyline plot
BTV	Bluetongue virus
CDV	Canine distemper virus
CPV	Capri pox virus
CMV	Cetacean morbillivirus
CDS	Coding sequence
c-H ELISA	Competitive H ELISA
CS	Constant size
CCPP	Contagious caprine pleuropneumonia
CPE	Cytopathic effect
dpi	Days post infection
dpc	Days post-challenge
dpv	Days post-vaccination

DCs	Dendritic cells
ddNTPs	Dideoxy terminators
DIVA	Differentiation between infected and vaccinated animals
DAM	DNA adenine methylase
DMV	Dolphin morbillivirus
PKR	Protein kinase R
DMEM	Dulbecco's modified Eagle's medium
EM	Electron microscopy
ER	Endoplasmic reticulum
EG	Exponential growth
FmoPV	Feline morbillivirus
FCS	Foetal calf serum
FAO	Food and agriculture organization
F	Fusion protein
GTR	General time-reversible
GP	Genome promoter
GFP	Green fluorescent protein
H	Haemagglutinin protein
HPD	Highest posterior density
hpi	hour post-infection
IFN	Interferon
IG	Intergenic
ICTV	International Classification and Taxonomy of Viruses

LAMP	Loop-mediated isothermal amplification
L	Large polymerase protein
LB	Luria Bertani
MCMC	Markov chain Monte Carlo
M	Matrix protein
MCC	Maximum clade credibility
MV	Measles virus
MDA5	Melanoma differentiation-associated protein 5
mAb	Monoclonal antibody
MOI	Multiplicity of infection
NA	Not applicable
NDV	Newcastle disease virus
dN	non-synonymous nucleotide substitutions
N	Nucleoprotein
nt	Nucleotide
OIE	World organisation for animal health/ Office International des Epizooties
ORF	Open reading frame
OD	Optical density
OPD	Ortho-phenylene diamine
PFA	Paraformaldehyde
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors

PI	Percent inhibition
PBMCs	Peripheral blood mononuclear cells
PPR	Peste des petits ruminants
PPRV	Peste des petits ruminants virus
PBS	Phosphate buffered saline
P	Phosphoprotein
PWMV	Pilot whale morbillivirus
PMV	Porpoise morbillivirus
PPD	Posterior probability distribution
qPCR	Quantitative real-time polymerase chain reaction
RACE	Random amplification of cDNA ends
rFP	Recombinant fowl pox
RBC	Red blood cells
RIG-I	Retinoic acid-inducible gene I
RLR	Retinoic acid-inducible gene I -like receptor
RT-PCR	Reverse-transcription polymerase chain reaction
RNP	Ribonucleoprotein
RVFV	Rift valley fever virus
RPV	Rinderpest virus
RdRP	RNA-dependent RNA polymerase
SLAM	Signalling lymphocyte activation molecule
SLAC	Single-likelihood ancestor counting
SDM	Site-directed mutagenesis

dS	Synonymous nucleotide substitutions
TMRCA	Time to most recent common ancestor
TCRV	Tissue culture rinderpest vaccine
TLRs	Toll-like receptors
TAE	Tris-acetate EDTA
UCED	Uncorrelated exponential distribution
UCLD	Uncorrelated lognormal distribution
UAE	United Arab Emirates
UTR	Untranslated region
VDS	Vero Dog SLAM
vcRNA	Viral complementary RNA
VLP	Virus like particle
VNT	Virus neutralisation test
VSP	Virus specific primer
WBCs	White blood cells

Chapter 1 General introduction

1.1 Background information

Peste des petits ruminants (PPR) is also known as 'goat plague', 'Kata', 'syndrome of stomatitis-pneumoenteritis' or 'ovine rinderpest'. PPR is an important infectious viral disease of domestic and wild small ruminants that threatens the food security and sustainable livelihood of farmers across Africa, the Middle East and Asia (Banyard *et al.*, 2010; Dhar *et al.*, 2002; Libeau *et al.*, 2014; Parida *et al.*, 2007). PPR is emerging in new regions of the world and is causing great economic losses (Perry *et al.*, 2002; Singh *et al.*, 2014; Stem, 1993). The causative agent, peste des petits ruminants virus (PPRV) belongs to the family *Paramyxoviridae*, genus *Morbillivirus* alongside other important viral pathogens such as rinderpest virus (RPV), measles virus (MV) and canine distemper virus (CDV). The live attenuated PPRV vaccine strains Nigeria 75/1 and Sungri 96 have been used successfully in the field for decades (Diallo *et al.*, 2007; Sen *et al.*, 2010). The commercially available diagnostic ELISAs are targeted against the nucleoprotein (N) and haemagglutinin (H) proteins and detect antibodies in vaccinated as well as naturally infected animals. No tools currently exist that allow serological Differentiation between Infected and Vaccinated Animals (DIVA). To this end, marker vaccines are a potential solution to the DIVA concept that may play an important role in the reduction of PPRV in endemic regions.

1.2 Taxonomy

PPRV belongs to the genus *Morbillivirus*, sub family *Paramyxovirinae*, family *Paramyxoviridae,* and order *Mononegavirales*. A detailed classification including other members of the order is provided in Table 1.1.

Table 1.1 Classification of the order *Mononegavirales*

Order	Family	Subfamily	Genus
Mononegavirales	*Bornaviridae*		*Bornavirus*
	Filoviridae		*Cuevavirus* *Ebolavirus* *Marburgvirus*
	Nyamiviridae		*Nyavirus*
	Rhabdoviridae		*Cytorhabdovirus* *Ephemerovirus* *Lyssavirus* *Novirhabdovirus* *Nucleorhabdovirus* *Perhabdovirus* *Sigmavirus* *Sprivivirus* *Tibrovirus* *Tupavirus* *Vesiculovirus*
	Paramyxoviridae	*Pneumovirinae*	*Metapneumovirus* *Pneumovirus*
		Paramyxovirinae	*Aquaparamyxovirus* *Avulavirus* *Ferlavirus* *Henipavirus* *Respirovirus* *Rubulavirus* ***Morbillivirus***

The morbillivirus genus contains seven species including PPRV (Figure 1.1) with classification being based on the requirements of the International Classification and Taxonomy of Viruses (ICTV).

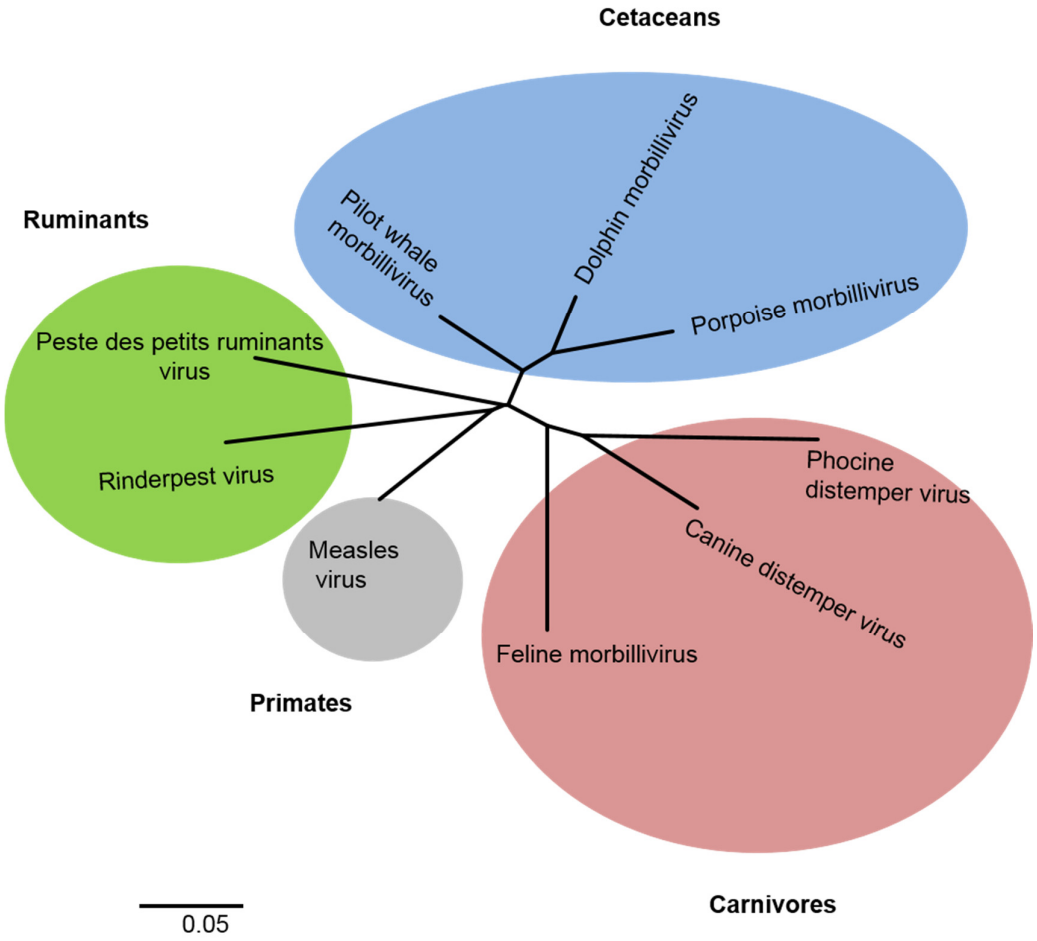

Figure 1.1 Un-rooted neighbour-joining tree showing the relationships between the different morbilliviruses. The phylogenetic tree was constructed using partial nucleoprotein gene sequences of 230 nucleotides (accession no NC_006383, peste des petits ruminants virus; NC_001498, measles virus; AB547189, rinderpest virus; NC_001921, canine distemper virus; KC802221, phocine distemper virus; JQ411016, Feline morbillivirus; AY949833, porpoise morbillivirus; NC_005283, dolphin morbillivirus and AF200818, pilot whale morbillivirus) with 1000 bootstrap replicates and the Kimura 2-parameter model in MEGA 5.2. The scale bar indicates nucleotide substitutions per site. Three virus strains, porpoise morbillivirus, dolphin morbillivirus, and pilot whale morbillivirus have been isolated from cetaceans are considered part of the species cetacean morbillivirus.

Three well characterised strains porpoise morbillivirus (PMV), dolphin morbillivirus (DMV) and pilot whale morbillivirus (PWMV) are considered part of the species cetacean morbillivirus (CMV) were isolated from different marine mammals. (Barrett *et al.*, 1993b; Taubenberger *et al.*, 2000). Historically measles virus (MV) and rinderpest (RPV) have been recorded for centuries as the cause of severe epidemics in humans or cattle, respectively, although the latter has been successfully controlled and globally eradicated. Canine distemper virus (CDV) was initially thought to be restricted to the infection of dogs although the virus has since been recorded in a number of terrestrial carnivore species including tigers, lions, hyena and non-human primates alongside infection of aquatic mammals (Buczkowski *et al.*, 2014). Recently, a completely new morbillivirus, feline morbillivirus (FmoPV), has been reported in domestic cats (Woo *et al.*, 2012).

Morbilliviruses are largely characterised at the molecular level through studies with the prototype virus, MV and to some extent CDV and RPV. PPRV is largely unexplored for virus replication and transcription. However, the morbilliviruses are known to be conserved across genus and family sharing similar characteristics. The description of PPRV in the sections below are generalised for morbilliviruses or paramyxoviruses with the inclusion of specific literature available to PPRV.

1.3 Virology

1.3.1 Virus structure

The viral genome for all members of the *Mononegavirales* consists of a single-strand of non-segmented negative-sense RNA encapsidated by N as a helical nucleocapsid.

The N-RNA complex, along with the RNA-dependent RNA polymerase (RdRP, large polymerase; L) and the co-factor phosphoprotein (P) forms a complex as ribonucleoprotein (RNP) complex (Figure 1.2). RNPs are contained within the virus envelope and appear as helical structures with a 'herring-bone' appearance. During the viral budding process, the viral envelope is derived from the infected cell membrane and is embedded with protruding viral fusion (F) and H glycoproteins (Figure 1.2). The matrix (M) protein is located on the inner surface of the envelope and bridges the RNPs and cytoplasmic tails of the membrane glycoproteins. Structurally, the morbilliviruses are pleomorphic (200-700 nm) enveloped particles as determined using negative-stain electron microscopy (EM) (Plowright *et al.*, 1962). No good EM structure for PPR virion is available to determine its structure and size. Morbillivirus particles contain more than one RNP and are therefore functionally polyploid in nature (Rager *et al.*, 2002).

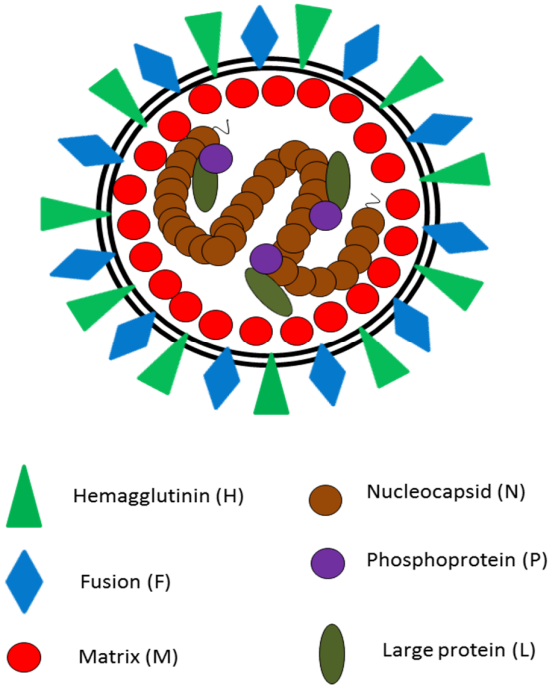

Figure 1.2 A schematic diagram of the PPR virion structure. The PPRV glycoproteins (F and H proteins) are embedded within the viral envelope. The M protein lines the inner surface of virus envelope. The ribonucleoprotein complex is composed of N, P and L proteins in association with the RNA genome.

1.3.2 PPRV genome organisation

The PPRV genome is a non-segmented, single-stranded, negative-sense RNA molecule of 15,948 nucleotides in length (Bailey *et al.*, 2005). The genome length of PPRV conforms to the 'rule of six' (multiple of six) like several other paramyxoviruses (Calain and Roux, 1993), as required for efficient genome replication and virus propagation (Bailey *et al.*, 2007) assuming that each N protein is bound to six nucleotides of genome or anti-genome RNA to form the RNP complex. The genome organisation of PPRV is presented in Figure 1.3.

The PPRV genome consists of six transcriptional units located in the order 3' N, P, M, F, H and L 5' that encode for the corresponding six structural proteins, N, P, M, F, H and L (Bailey *et al.*, 2005; Chard *et al.*, 2008) and two additional non-structural proteins, C and V that are translated via alternative mechanisms from the P mRNA (Mahapatra *et al.*, 2003). A breakdown of the PPRV genome sequence and its component parts, including their position and length, is presented in Table 1.2. Transcriptional units are separated from each other by an intergenic (IG) trinucleotide sequence that for the morbilliviruses consist of the sequence, CTT. The region upstream of the IG region includes a polyadenylation signal for the upstream gene and the sequence after the IG region marks the 5' transcriptional start site of the next transcriptional unit. The IG trinucleotides are not transcribed during the transcription of individual genes but are incorporated by the RdRP into the positive strand replicative intermediate during the replicative stage of the viral life cycle.

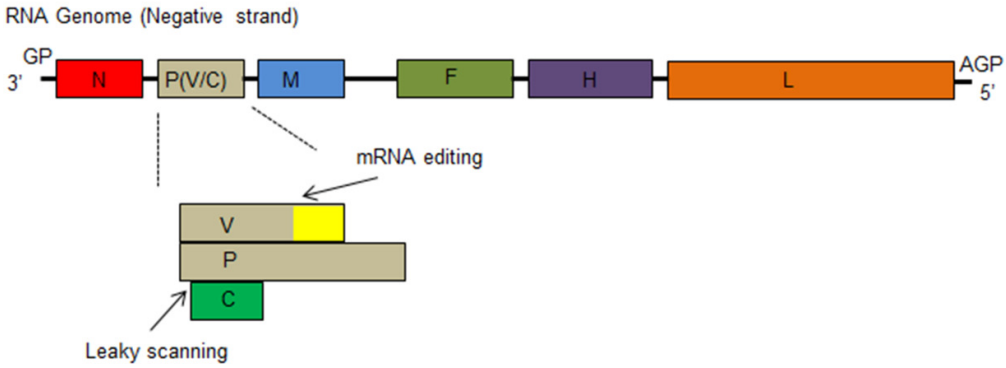

Figure 1.3 A schematic representation of the PPRV genome organisation. The PPRV genome is a non-segmented, single-stranded negative sense RNA molecule. The genome consists of six transcriptional units (encoding the nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin (H) and large/ polymerase (L) proteins) that are flanked by a 3' genome promoter (GP) and a 5' anti-genome promoter (AGP). The P transcriptional unit also encodes for additional C and V non-structural proteins. The V protein is produced by co-transcriptional P mRNA editing by insertion of a non-template G residue and the C protein is produced from the alternative reading frame in P mRNA by leaky scanning during translation.

A 3’ leader and a 5’ trailer terminal sequence are present at the genome termini. These are largely complementary over the first 20 nucleotides and contain conserved binding sites for the RdRP. As such these short untranslated regions play an important role as regulatory elements in replication, transcription and packaging of RNA genome during virus propagation (Bailey *et al.*, 2007). For RPV, mutations introduced into the 3’ genome promoter region greatly affected the promoter activity in a minigenome assay (Mioulet *et al.*, 2001) and exchange of the genome promoter (GP) and anti-genome promoter (AGP) regions between virulent and avirulent strains of recombinant RPV affected the replicative ability of the virus with a virulent strain of RPV containing vaccine GP and AGP being strongly attenuated (Banyard *et al.*, 2005).

Untranslated regions between transcriptional units can be of a variable length. In particular the 3’ untranslated region (UTR) of the M gene (444 nucleotides) and 5’ UTR of the F gene (633 nucleotides) are considerably longer in length than the other UTRs in the viral genome. The UTR between the M and F genes is rich in GC nucleotides (68 - 72%). Generally, between isolates the PPRV genome is relatively conserved with a maximum divergence reported of 12% at the nucleotide level and 7% at the amino acid sequence level.

Table 1.2 The genome organisation of PPRV

Sequence components	Nucleotide Position on genome	Total length	5'UTR	CDS	3'UTR	Intergenic region, nucleotide	Protein Size, amino acid	Deduced MW, KDa
Leader	1-52	52	NA	NA	NA	(CTT)	NA	NA
N mRNA	55-1744	1689	52	1578	59	CTT	525	58
P mRNA	1748-3402	1655	59	1530	66	CTT	509	55
V (P) mRNA	1748-3402	1656	59	897	700	CTT	298	31
C (P) mRNA	1748-3402	1655	81	534	1040	CTT	177	20
M mRNA	3406-4888	1484	32	1008	444	CTT	335	38
F mRNA	4892-7302	2410	633	1641	136	CTT	546	59
H mRNA	7306-9262	1957	20	1830	107	CTT	609	69
L mRNA	9266-15908	6643	22	6552	69	(CTA)	2183	247
Trailer	15912-15948	37	NA	NA	NA	NA	NA	NA

* CDS, coding sequence; UTR, untranslated region; ORF, open reading frame; NA, not applicable.

1.3.3 PPRV entry, replication cycle and packaging

The replication cycles of all paramyxoviruses are similar and the first step is the attachment of the virus to a cell surface and membrane fusion to release a genome into a cell cytoplasm in a pH-independent manner (Figure 1.4). Like other morbilliviruses, PPRV has an established lymphatic and epithelial tropism (Birch *et al.*, 2013; Couacy-Hymann *et al.*, 2007b). The H protein is responsible for the attachment of a virus to the cell surface through the recognition of host cell receptor molecules such as sialic acid, immune cell marker signalling lymphocyte activation molecule (SLAM)/ CD150 (Adombi *et al.*, 2011; Seki *et al.*, 2003) or the recently reported epithelial cell receptor Nectin-4 (Birch *et al.*, 2013).

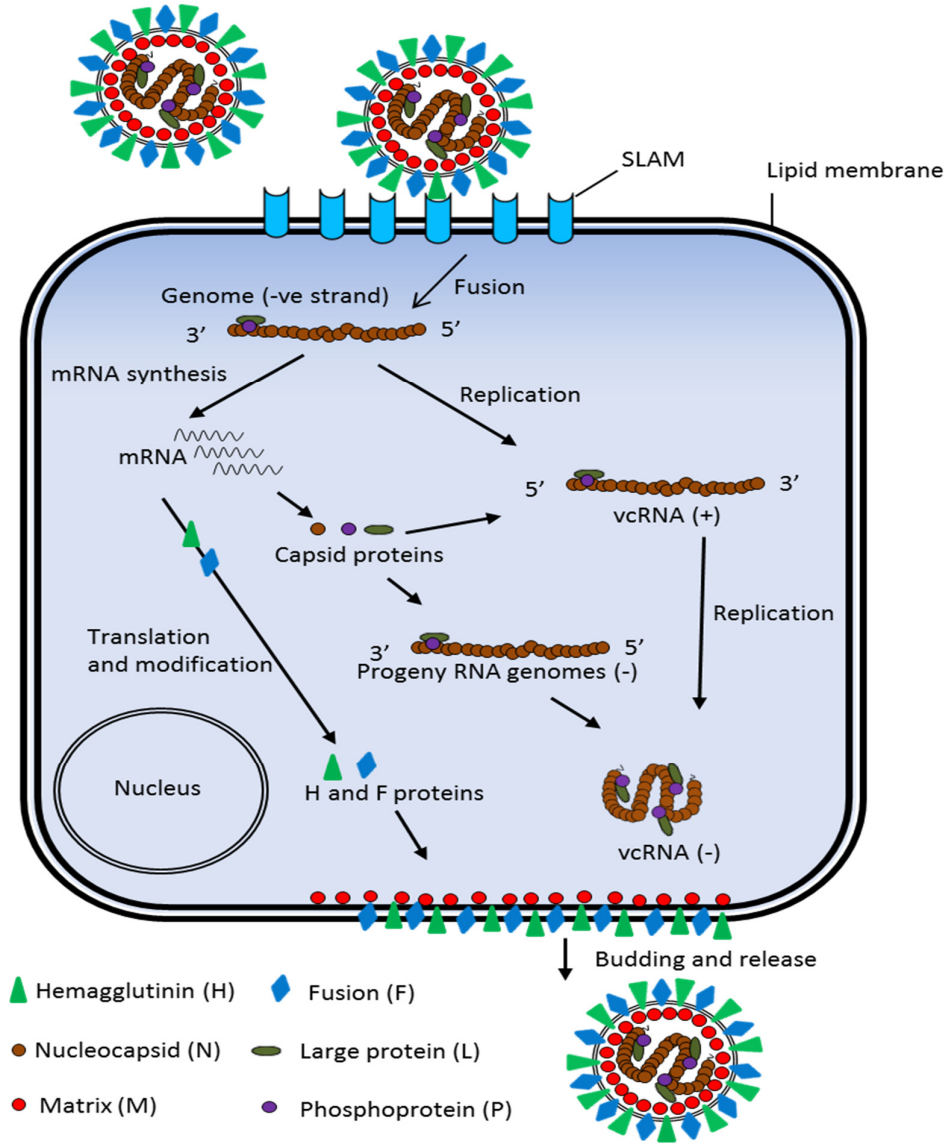

Figure 1.4 A schematic replication of life cycle of a morbillivirus. Adapted from (Moss and Griffin, 2006). The first step in virus infection is the attachment of a virion to a host cell surface receptor which leads to the fusion of viral and cellular membrane. The negative sense RNA genome is released into the cell cytoplasm and transcription initiates to produce viral gene transcripts. Later, following the production of viral proteins a switch to a replicative mode occurs that results in the production of a positive sense anti-genome RNA, a replicative intermediate which as a template for the generation of nascent genome RNA. The viral transcripts are translated using host cell translational machinery and later viral components are assembled and budded in to new virion.

For binding to SLAM, an interaction between the V domain of SLAM and the β-sheet 5 quartet of the H protein leads to conformational changes in the H protein (Navaratnarajah *et al.*, 2008). The H protein conformational change upon receptor binding triggers the F protein to initiate membrane fusion. It has been postulated that the morbillivirus H and F proteins are associated (the H dimer with the F_1-F_2 trimer) prior to receptor attachment thus preventing premature fusion by the F protein. Attachment of the H protein to receptors creates a scaffold and later activates the F protein to function and drive fusion of the viral envelope with the host cell membrane and release the RNP complex into the cell cytoplasm (Ludwig *et al.*, 2008; Plemper *et al.*, 2002). Morbilliviruses replicate exclusively in the cytoplasm of host cells although it is not clear whether replication occurs diffusely throughout the cell cytoplasm or whether there are dedicated intracellular sites within cytoplasm where replication occurs (Duprex *et al.*, 1999). The genomes of morbilliviruses are never found as naked RNA and are always bound to N protein to form helical RNP complex, an interaction that confers resistance to host cell RNases. The RNP consists of the negative sense (–) genome or a positive sense anti-genome (+) RNA. The RNP complex containing the RNA, with the N, P and L proteins makes up the minimal replicative unit of these viruses that is required for the initiation of the transcription and replication processes. The L protein contains RdRP activity and with the co-factor P protein, functions in both transcription and replication of the genome. The RdRP uses the RNP as a template and cannot act on naked RNA (Rozenblatt *et al.*, 1979). The interaction of the RdRP with RNA is mediated by the P protein which has both L and P binding sites (Kingston *et al.*, 2004; Longhi *et al.*, 2003). A viral genome as a negative sense RNP is released into the cell cytoplasm following fusion of the viral and cellular membrane and is acted upon by viral polymerase complex

that enters at GP and transcribes the viral genes (Horikami and Moyer, 1991). During the transcriptional mode, it is not clear whether the RdRP always produces a 3' leader (52 nucleotide) sequence or whether it directly transcribes the capped mRNA of the N gene. As the polymerase reaches the IG regions it may fall off the template or continue to transcribe the next gene. If the polymerase detaches from the template it can only reinitiate at the GP and as such a transcriptional gradient is produced whereby the 3' proximal genes are produced in abundance with a reduction in gene expression along the genome (Rennick *et al.*, 2007). Thus, the fine control in producing required relative amounts of each viral protein is achieved through the transcriptional gradient. The viral mRNAs produced are 5' methylated and 3' poly-adenylated by the viral polymerase and are translated by host cell translation machinery (Banerjee, 1987).

Post-translational modification of the viral glycoproteins (H and F) is carried out on the endoplasmic reticulum (ER) as proteins are transported to the cell membrane via the Golgi apparatus. At a later stage following infection, the polymerase switches from a transcriptive to replicative state and its been hypothesised that the switch is mainly due to the accumulation of a sufficient intracellular concentration of the N and P proteins required for encapsidation of genomic RNA (Banerjee, 1987; Lamb and Kolakofsky, 2001). During the replication mode the signals of the gene start, IG and gene stop regions are ignored and a full-length anti-genome RNA is produced including a leader sequence. Later, to generate a nascent genome strand RNA, the polymerase enters the AGP in the newly synthesised positive sense anti-genome RNA template to produce genome RNA. Unlike the mRNA, the nascent genome or

anti-genome RNA molecules produced are not capped or polyadenylated and are immediately encapsidated by the N protein.

Synthesis of nascent genomes and viral components to a sufficient amount in the host cell leads to the assembly and budding of virus particles. The interaction between the various viral components during transportation to the cell membrane for assembly and packaging into new virus particles is not well understood. The M protein is believed to play a role in the assembly of the viral RNA and protein components into a host cell membrane, and packaging, budding and release of new virion particles (Takimoto and Portner, 2004). The M protein interacts with both RNPs and the cytoplasmic domains of the glycoproteins at the plasma membrane and thus forms the bridge between them. The interaction between the M protein and glycoproteins is specific to each virus. Where studies have attempted to examine these interactions are requirement for homologous interactions have been reported. For example, a chimeric RPV with the glycoproteins replaced from PPRV was viable but grew to a low titre (Das *et al.*, 2000) and it was found that the addition of the homologous M protein from PPRV improved the titre of the rescued virus (Mahapatra *et al.*, 2006). Functionally, the M protein has been shown to inhibit RNA transcription and interact with the N protein to assemble into RNPs and transport to the cell membrane (Iwasaki *et al.*, 2009; Runkler *et al.*, 2007).

1.3.4 PPRV proteins

1.3.4.1 Nucleoprotein (N)

The N gene is the first to be transcribed and being located at 3' proximal end of the genome expresses the most abundantly produced mRNA. The N protein of PPRV is

525 amino acids in length (58 KDa) and consists of a conserved N-terminal structural core domain (N_{CORE}) and a highly variable C-terminal domain (N_{TAIL}) (Bodjo *et al.*, 2008; Diallo *et al.*, 1994). The primary function of the N protein is to encapsidate the genomic RNA to form a helical nucleocapsid structure, resistant to RNase degradation (Lamb and Kolakofsky, 2001). The nucleocapsid structure appears to be a 'herringbone' structure by negative-stain EM (Barrett *et al.*, 1993a; Bodjo *et al.*, 2008).

Each N protein binds and encapsidates exactly six nucleotides of RNA (Calain and Roux, 1993; Kolakofsky *et al.*, 1998) and therefore 2658 (=15948/6) N proteins are required per genome of PPRV for complete encapsidation. The large amount of N protein required for encapsidation is likely the reason for its 3' terminal genome location. N protein monomers, aided by P (serving as a molecular chaperone) prevent N-N aggregates from self-assembling and this soluble form of N-P is the substrate for encapsidation of nascent RNA. The encapsidated RNA retains considerable conformational flexibility to allow transcription and replication (Figure 1.5). The association of N and P is also essential for the functioning of the L protein in RNA synthesis in MV (Buchholz *et al.*, 1994; Ryan and Portner, 1990).
The N protein of the morbilliviruses is also highly antigenic in nature and induces an N protein specific immunity that does not confer protection (Lamb and Kolakofsky, 2001).

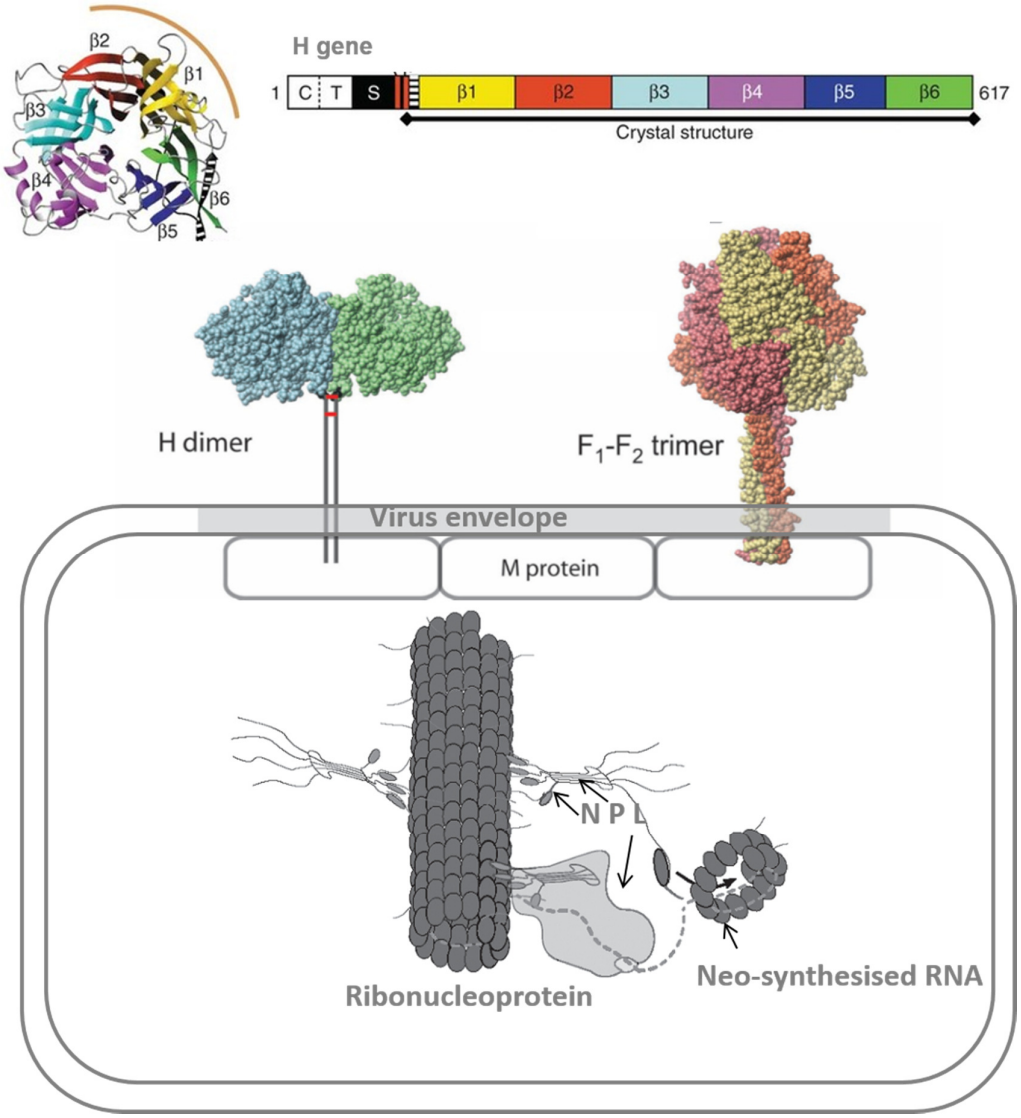

Figure 1.5 The structural proteins of paramyxoviruses. Crystal structures of measles virus H (Hashiguchi *et al.*, 2007) and parainfluenza virus 5 F (Yin *et al.*, 2006) proteins attached to cell membrane are shown as a space-filled model. The ribbon plot model (left side of H dimer) of a six-bladed β-propeller sheet H-protein head structure and location in the H protein of measles virus is shown at the top (Navaratnarajah *et al.*, 2011). The M protein, located on the inner side of virus envelope, bridges with ribonucleoprotein complex containing the N, P and L proteins with the polymerase activity being shown below (Griffin and Oldstone, 2009). The figure has been generated with elements adapted from the various sources as cited within the caption.

1.3.4.2 Phosphoprotein (P)

The P protein is encoded by the second gene and consists of 509 amino acids with a predicted size of 55 KDa. It is genetically the least conserved protein with short interspersed conserved domains (Barrett and Underwood, 1985). The P protein is heavily phosphorylated, in a process mediated by cellular kinases. The active form of P protein is a tetramer and it is a multi-functional protein which interacts with both the N and L protein as N-P and P-L complexes (Longhi et al. 2003; Kingston et al. 2004). As described, P protein plays a chaperone role preventing excessive N protein self-assembly, maintaining its soluble state and helping in the formation of the nucleocapsid structure. The P protein acts as co-factor for the L protein in the progression of the RdRP complex on the RNA template during replication.

1.3.4.3 Matrix (M) protein

The M protein is 335 amino acids (38 KDa) in length and is the most conserved protein among PPRV isolates. The M protein is believed to be involved in assembly and transport of viral components to the apical surface of host cell membrane and budding of virions (Takimoto and Portner, 2004). Within the virion it is located on the inner surface of the virus envelope, where it interacts with the RNP and the cytoplasmic domains of H and F glycoproteins embedded in the envelope (Baron *et al.*, 1994) (Figure 1.5). Recombinant viruses lacking the M protein are unable to assemble and bud efficiently reiterating its role in these elements of the viral life cycle (Pantua *et al.*, 2006). Recently, the co-expression of the PPRV M, H and N proteins in Sf9 insect cells has been reported to enable generation of PPRV virus-like particles (Liu *et al.*, 2014a; Liu *et al.*, 2014b).

The extended UTR between the M and subsequent F gene described earlier is proposed to play a role in regulating translation by forming secondary structures in M and F mRNAs (Bailey *et al.*, 2005; Dhar *et al.*, 2006; Meyer and Diallo, 1995). A recombinant MV lacking the long M/F UTR demonstrated that the deleted region was not essential for viral replication, although its absence altered the viral phenotype (Takeda *et al.*, 2005). In addition, the nine-nucleotide sequence in the 5' UTR of PPRV F gene was found to be complementary to the 18S ribosomal RNA (Chulakasian *et al.*, 2013).

1.3.4.4 Fusion (F) protein

The F protein consists of 456 amino acids and is a type I transmembrane glycoprotein/ fusion protein, and is responsible for virus-host cell fusion and infected cell-cell fusion. Fusion activation needs the synergistic effect of the H protein (Figure 1.5) (Lamb, 1993; Morrison, 2003). The F protein is synthesised as an inactive precursor polypeptide (F_0) with a cleavable N-terminal 28 amino acid signal peptide. The signal peptide directs the transport of protein to the endoplasmic reticulum for trimerisation (Plemper at al., 2001). The F_0 trimers are cleaved by cellular furin in the trans-Golgi at a conserved R-X-R/ K-R site (Chard *et al.*, 2008). Cleavage results in the generation of large (F_1) and small (F_2) protein components that form an active heterodimer a linked by disulphide bonds. The un-cleaved F_0 trimers transported to the virus envelope may also be cleaved by extracellular host cell enzymes. The new N-terminus of the F_1 protein (20-25 amino acids) is hydrophobic, and acts as a fusion peptide. This peptide is inserted into the target membrane during the fusion process. The C-terminal ends of the F_1 and F_2 peptides

have transmembrane domain functions in membrane anchoring (Meyer and Diallo, 1995; Rahaman *et al.*, 2003).

1.3.4.5 Haemagglutinin (H) protein

The H protein consists of 609 amino acids and is a type II glycoprotein, where the C-terminus of the H protein extends outside the cell membrane and the N-terminal transmembrane domain extends towards the cytoplasmic milieu. Unlike other morbillivirus H proteins, the PPRV H displays both haemagglutination activity (agglutination of erythrocytes) and neuraminidase activity (cleaves sialic acid residues from the carbohydrate moieties of glycoproteins) (Dhar *et al.*, 2006; Seth and Shaila, 2001) and as such is often termed the HN protein. The MV H protein shows haemagglutination activity but lacks neuraminidase activity (Varsanyi *et al.*, 1984) whilst the RPV H protein lacks both haemagglutination and neuraminidase activity (Langedijk *et al.*, 1997).

The H protein displays a high degree of antigenic variability among the morbilliviruses due to immunological pressure as it carries B cell epitopes that generate neutralising antibodies (Renukaradhya *et al.*, 2002). The cell surface receptor, SLAM (CD150), binding site was mapped for the H protein of PPRV (Vongpunsawad *et al.*, 2004). The crystal structure of the MV H protein has been determined (Hashiguchi *et al.*, 2007) and has shown that each monomer of a dimer of H protein exhibits a six-bladed β-propeller head structure and a connecting membrane stalk (Figure 1.5). The H protein binding to the receptor molecule

transmits the fusion-triggering signal to the F protein to initiate membrane fusion for MV entry (Navaratnarajah *et al.*, 2011).

1.3.4.6 Large (L) / polymerase protein

The L protein is the largest PPRV protein (247 KDa) and is encoded by a gene (2,183 amino acids) located at the 5' proximal end of the genome. The L gene accounts for 40% of the total genome length. The L protein is the least abundant protein produced as it is required in catalytic amounts due to its polymerase enzymatic activity. The L protein, and its RdRP activity, is essential in both the replication and transcription of genes including the capping and polyadenylation of viral mRNAs (Hammond *et al.*, 1992; Hercyk *et al.*, 1988; Horikami *et al.*, 1992). The P protein is an essential co-factor of the L protein during polymerase activity. L protein sequences and their different functions are highly conserved across the paramyxoviruses with clearly identified protein domains (McIlhatton *et al.*, 1997). The L protein consists of three large conserved domains connected by variable hinge regions (McIlhatton *et al.*, 1997). The first N-terminal domain is involved in interactions with both the N and P proteins whilst the second domain carries out phosphodiester bond formation during polymerase activity. Finally the third domain has an ATP binding function facilitating kinase activity (Blumberg *et al.*, 1988). Studies have shown that the green fluorescent protein (GFP) ORF can be inserted into the second variable hinge region of the L protein of recombinant MV, CDV and RPV without significantly disrupting the function of the L protein (Brown *et al.*, 2005; Duprex *et al.*, 2002). These recombinant viruses were used to study virus pathogenesis and suggested inclusion of a heterologous protein into the L ORF as a

means of functional attenuation of these viruses (Brown *et al.*, 2005; Duprex *et al.*, 2002; Silin *et al.*, 2007).

1.3.4.7 Non-structural V and C proteins

The C and V proteins are non-structural or accessory proteins produced from the P ORF by alternative translational and transcriptional mechanisms (Figure 1.3). The V protein is encoded from an overlapping ORF within the P mRNA. The P mRNA transcription initiates normally and at position 751, a pseudo-template addition of one G nucleotide residue occurs due to viral RdRP slippage at the editing site complex. During translation, this leads to frame shift downstream of the mRNA editing site to produce the V protein of 298 amino acids (Mahapatra *et al.*, 2003). An identical RNA editing mechanism has been reported in other morbilliviruses (Barrett *et al.*, 1993a; Cattaneo *et al.*, 1989) and although less frequent, the generation of V transcripts accounts for one third of the total mRNA generated from the P gene start codon in MV (Cattaneo *et al.*, 1989). The V/ P proteins share identical amino-terminus sequences but have a different C-terminal region due to the frame shift. Surprisingly, the common V/ P N-terminus is poorly conserved whereas the carboxyl-terminal region of V protein is highly conserved and rich in cysteine residues with zinc binding motifs (Mahapatra *et al.*, 2003).

The C protein is encoded by the P mRNA through utilisation of an alternative reading frame (+1); beginning 23 nucleotides downstream of the P protein start codon (Figure 1.3). The C protein is produced by leaky ribosomal scanning of P mRNA during the translational process. The C protein of PPRV consists of 177

amino acids and has a molecular weight of 20 KDa (smaller than V protein). C is the least conserved protein across all of the morbilliviruses.

The V and C proteins of morbillivirus have been reported to act as virulence factors modulating the host cell innate immune response (Patterson *et al.*, 2000). The V and C proteins are also required for normal virus propagation as they regulate transcription and replication during virus infection. The V protein of RPV has been reported to interact with both the N and L proteins, and is therefore involved in both transcription and replication (Sweetman *et al.*, 2001). A recombinant knockout RPV that lacked the expression of either V or C proteins individually or in tandem had impaired growth *in vitro* in B95a cells (Baron and Barrett, 2000). The V knockout RPV had an increased level of genome and anti-genome synthesis that led to change in cytopathic effect (CPE) to a more syncytium-forming phenotype (Baron and Barrett, 2000). The C knockout RPV was attenuated with a measurable reduction in viral mRNA synthesis. RPV lacking both V and C protein expression had a cumulative effect with severe growth defects (Baron and Barrett, 2000). Similarly, a C and V deficient wild-type MV was found to be attenuated in monkeys (Devaux *et al.*, 2008). Overall, paramyxovirus accessory proteins are involved in the modulation of the host cell mediated immune response generally through limiting the cellular interferon (IFN) response (section 1.7). A recent study with PPRV demonstrated that the V protein blocks type I IFN action and partially blocks type II INF action (Chinnakannan *et al.*, 2013).

1.4 Disease

1.4.1 Clinical signs

Goats and sheep are the primary host for PPRV. Goats are more susceptible to disease than sheep (Nanda *et al.*, 1996). As described in the OIE (world organisation for animal health/ Office International des Epizooties) manual (OIE, 2012), the incubation period is typically 4 to 6 days although it may range between 3 and 10 days. At the acute stage of disease, animals show pyrexia up to 41°C lasting for 3 to 5 days, depression, anorexia and dryness in the muzzle. Watery nasal and lachrymal discharges gradually become mucopurulent with excessive salivation. Erosive lesions formed in the oral cavity may become necrotic. In the later stage of infection, animals develop diarrhoea, coughing with laboured abdominal breathing. The disease condition may last for 14 days before recovery from infection or leads to death during the acute stage of infection. The morbidity rate can reach 100% with a high case fatality with the acute form of disease. The above described clinical signs and mortality can vary considerably depending on the virulence of virus (OIE, 2012).

1.4.2 Pathogenesis

PPRV pathogenesis is poorly understood and knowledge has been gained mainly by comparison with closely related RPV (Brown and Torres, 1994; Wohlsein *et al.*, 1993). The study of pathogenesis of PPRV has mainly been described through the experimental infection of small ruminants with a virulent virus to develop a reliable and reproducible animal model (Alcigir *et al.*, 1996; Baron *et al.*, 2014a; Bundza *et al.*, 1988; El Harrak *et al.*, 2012; Hammouchi *et al.*, 2012; Pawaiya *et al.*, 2004; Pope *et al.*, 2013; Truong *et al.*, 2014). Histological investigation during early infection

showed immune cell driven spread of PPRV similar to that seen with other morbilliviruses (Pope *et al.*, 2013). The initial site for virus replication was observed within the tonsillar tissue and lymph nodes draining the site of inoculation. It has been proposed that the virus infected immune cells within the respiratory mucosa migrate to the local lymphoid tissue where primary virus amplification occurs and from which virus enters the general circulation. Clinical signs usually develop at 3-4 days post infection (dpi) with pyrexia and anorexia. Anorexia results from the presence of lesions in the buccal cavity, tongue and dental jaws which make mastication uncomfortable. Leucopoenia is often observed from 4 dpi with a considerable reduction of $CD4^+$ T cells (Baron *et al.*, 2014a; Herbert *et al.*, 2014). Disease progresses with lacrimal, nasal and mucosal discharges with live virus being detected in those excretions as early as 4 dpi. Viral antigens were also observed by histochemical staining in lymphoid organs, the respiratory and the gastrointestinal tracts (Kumar *et al.*, 2004; Pope *et al.*, 2013). Histopathological assessment of infected tissues observed a large number of syncytia in lymphoid tissue between 5 and 7 dpi that later become necrotic. In the later stage of infection, erosive lesions that become necrotic are seen in the oral cavity. The severity of clinical signs peaks between 6 and 8 dpi and signs continue for a maximum of 14 days before death or recovery from infection. Three visible forms (severe, mild and subclinical) of PPR are seen depending on the virulence of the infecting virus and several other predisposing factors such as nutritional state of the animal and the presence of secondary infections. However, despite the differentiation of PPRV isolates into genetically distinct lineages, no lineage specific differences in the pathogenicity of the virus were observed following experimental infection (Baron *et al.*, 2014a). Goats are considered to be more susceptible to disease with similar pattern of

pathological signs compared to sheep (Truong *et al.*, 2014) and even specific breeds of goats may be more susceptible than others (Couacy-Hymann *et al.*, 2007a).

1.5 Epidemiology

1.5.1 Origin and distribution

The first report of PPR was made in 1942 in the Ivory Coast (Gargadennec and Lalanne, 1942) based on the observation that a disease of small ruminants was not transmissible to in-contact cattle. After three decades, the causative agent of PPR disease was defined as a distinct viral entity (Gibbs *et al.*, 1979). Before 1942, it is likely that PPR would had been confused with rinderpest as they show similar clinical symptoms and several reports of epidemic rinderpest-like disease in small-ruminants in Senegal and French Guinea were published between 1871 and 1927 (Diallo, 1988). Contrastingly, infections of rinderpest with an absence of clinical disease in sheep and goats are well documented in the same regions of Africa (Rossiter *et al.*, 1983). The evolution of PPRV and its relationship with RPV has been dissected out recently using molecular phylogenetic analysis of virus genome sequence data. Bayesian phylogenetic studies found that the RPV is more closely related to MV than to PPRV (Furuse *et al.*, 2010; Pomeroy *et al.*, 2008). The origin of ancestral PPRV and its relation to other morbilliviruses and evolutionary changes are investigated further in this PhD study.

After the first recognition of PPR in Ivory Coast increased awareness led to further reports in neighbouring countries in the order, Senegal, Chad, Togo, Benin, Ghana,

Nigeria, Oman, Sudan, Saudi Arabia, India, Jordan, Israel, Ethiopia, Kenya, Uganda and Pakistan (Sen *et al.*, 2010). This appears to be the geographic spread of disease from West Africa to East Africa, and then to the Middle East and Asia. PPRV is considered to be endemic across Africa, the Middle East and Asia (Banyard *et al.*, 2010; Dhar *et al.*, 2002; Kwiatek *et al.*, 2011).

In recent years PPRV has extended its boundaries southwards in Africa as far as southern Tanzania (2008) and the Democratic Republic of Congo and Angola (2012). PPR outbreaks have also been reported across North Africa including within Tunisia (2006), Morocco (2008) and Algeria (2011). Alongside this, within Europe, Turkey reported approximately twenty laboratory confirmed PPR outbreaks in sheep and goats during 2011-2012. In southwest Asia, the virus spread to Tibet (2007) and has recently been reported all over China (2013-2014) (FAO, 2013).

The molecular epidemiology of PPR, based on the sequence comparison of a small 322 nucleotide (nt) region of the F gene (Forsyth and Barrett, 1995), 255 nt of the N gene (Couacy-Hymann *et al.*, 2002) or 298 nt of the H gene (Senthil Kumar *et al.*, 2014) has defined the existence of four distinct lineages (I-IV) of PPRV (Banyard *et al.*, 2010; Dhar *et al.*, 2002; Kwiatek *et al.*, 2011; Shaila *et al.*, 1996). The nomenclature of lineages I and II are slightly different for the N and F gene and is historically reversed depending on the assay used (lineage II of F gene named as lineage I of N gene). However, recently a comparison has been made between the F, N and H gene phylogenetic analyses and the partial N gene sequence was shown to be a more accurate predictor of viral diversity due to sequence variability across the region examined (Senthil Kumar *et al.*, 2014).

Historically, lineages I-III were found in Africa and numbered according to apparent spread of virus from West Africa (I and II) to East Africa (III). Lineage IV was mainly restricted to the Middle East and Asia with a few exceptions of Lineage III in Yemen and Oman and mixed lineages of III and IV in UAE and Qatar. However, lineage IV has now established its presence all across the PPR endemic areas with frequent outbreaks in Africa (Kwiatek *et al.*, 2011). PPRV lineages circulating in Africa (Table 1.3) and the Middle East and Asia (Table 1.4) are shown in Figure 1.6.

Table 1.3 Lineages of PPRV circulating in different countries of Africa, based on partial N/F gene sequence analysis (taken from Parida *et al*., 2015).

Country	Year of first report	Lineage	Year of confirmation of outbreak through sequencing	NCBI submission
Algeria	2010	IV	2010	Yes
Angola	2012	IV	2012	No
Benin	1972	NA	NA	No
Burkina Faso	NA	I	1988	Yes
		II	1999	No
Cameroon	NA	IV	1997	Yes
Central African Republic	NA	IV	2004	Yes
Chad	1971	II	1993	No
Comoros	2010	NA	NA	No
Congo	NA	IV	2006	No
Democratic Republic of the Congo	2012	IV	2012	No
Egypt	1987	IV	2009, 2010, 2012	Yes
Eritrea	NA	IV	2002, 2003, 2005, 2011	Yes
Ethiopia	1994	III	1994, 1996	Yes
		IV	2010	Yes
Gabon	NA	IV	2011	Yes
Ghana	NA	II	1976, 1978, 2010	Yes
Guinea	NA	I	1988, 1991	Yes
Guinea Bissau	NA	I	1989	Yes
Ivory Coast	1942	I	1989	Yes
Kenya	2006	III	2006	Yes
Libya	NA	NA	NA	No
Mali	NA	II	1999	Yes
Mauritania	NA	II	2012	Yes
Morocco	2008	IV	2008	Yes
Niger	NA	II	2012	No
Nigeria	1967	II	1975, 1976, 2010, 2012, 2013	Yes
		IV	2008, 2009, 2010, 2012, 2013	Yes
Senegal	1955	I	1964, 1994	Yes
		II	2010, 2013	Yes
Sierra Leone	2008	II	2009	Yes
Somalia	2006	III	NA	No
Sudan	1971	III	1971, 1972, 2000	Yes
		IV	2000, 2005, 2008, 2009	Yes
Tanzania	2008	III	2010, 2011, 2013	Yes
Togo	1972	NA	NA	No
Tunisia	NA	IV	2012, 2013	Yes
Uganda	1995	III	2007	No
		IV	2007, 2008	Yes
Western Sahara	NA	IV	2010	No

*Lineages of isolates of PPRV were named by following the classification of lineages based on partial N gene sequence phylogenetic analysis; NA, not available.

Table 1.4 Lineages of PPRV circulating in different countries of the Middle East and Asia, based on partial N/F gene sequence analysis (taken from Parida *et al*., 2015).

Country	Year of first report	Lineage*	Year of confirmation of PPR outbreak through sequencing	NCBI submission
Turkey	1996	IV	1996,2000, 2006, 2007, 2008, 2009, 2010, 2011	Yes
Iraq	1998	IV	2011, 2012, 2013	Yes
Iran	1995	IV	1998, 2010, 2011, 2012	Yes
Saudi Arabia	1980	IV	1999, 2004	Yes
Israel	1993	IV	1993, 1995,1998,	Yes
Jordan	NA	IV	NA	No
Kuwait	1999	IV	1999	No
Oman	1978	III	1983, 1987	Yes
Yemen	2002	III	2001, 2009	No
Qatar	NA	III IV	2010 2010	Yes Yes
UAE	1983	III IV	1986 NA	Yes No
Lebanon	2006	NA	NA	No
Afghanistan	1995	NA	NA	No
Kazakhstan	1997	NA	NA	No
Tajikistan	2004	NA	2004	Yes
India	1987	IV	1994, 1995, 1996, 1998, 1999, 2004, 2002, 2001, 2003, 2005, 2007, 2008, 2012	Yes
Pakistan	1991	IV	1994, 2005, 2006, 2007, 2008, 2009, 2010, 2012,	Yes
Bangladesh	1993	IV	2000, 2008, 2009, 2010, 2011, 2012	Yes
Nepal	1995	IV	1995, 2009	Yes
China	2007	IV	2007, 2008, 2014	Yes
Bhutan	2010	IV	2010	Yes
Vietnam	2007	Serology	NA	No

* Lineages of isolates of PPRV were named by following the classification of lineages based on partial N gene sequence phylogenetic analysis; NA, not available.

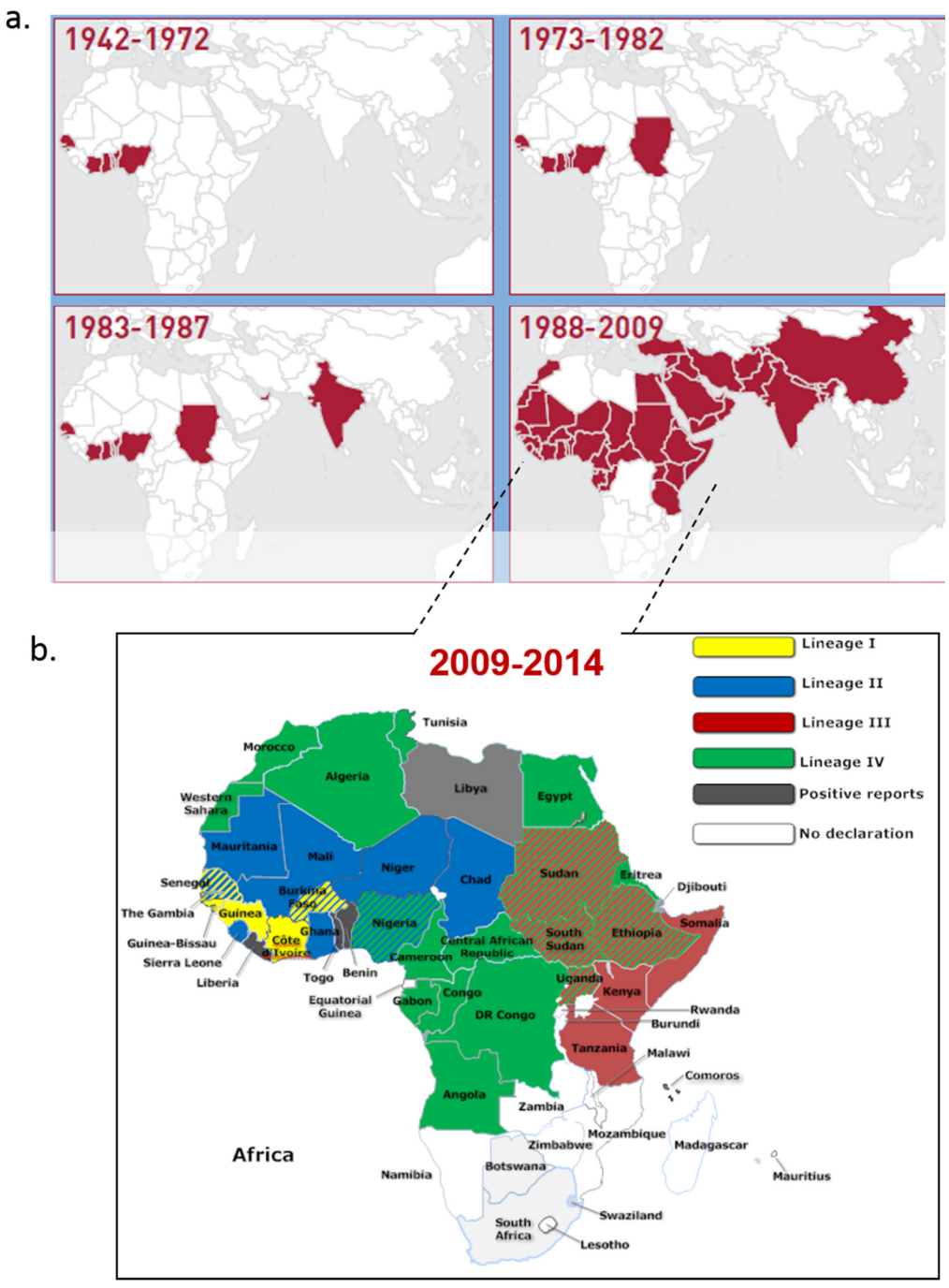

Figure 1.6 The global spread of PPRV from its first detection in 1942 to 2014. (a) depicts global distribution of PPRV from 1942 to 2009, taken from (FAO, 2009) and (b) depicts recent circulations of PPRV including lineage distribution in Africa and was drawn using smart draw software (USA).

1.5.2 Host range

Sheep and goats are the primary hosts for PPRV with a few reports of disease outbreaks in camels (Khalafalla *et al.*, 2010; Kwiatek *et al.*, 2011; Roger *et al.*, 2001; Saeed *et al.*, 2004) . Cattle (Anderson and McKay, 1994; Lembo *et al.*, 2013; Sen *et al.*, 2014), buffalo (Govindarajan *et al.*, 1997) and pigs (Nawathe and Taylor, 1979) develop subclinical infection with PPRV and are not thought to be capable of excreting virus and contributing to the disease epidemiology. The infection of wildlife, mainly living under semi-free range conditions, has also been reported (detailed in Table 1.5). The exact role of wildlife animals in the epidemiology of PPR disease remains to be clarified.

Infected animals can transmit PPRV to close in-contact susceptible animals through exhaled aerosols, particularly during coughing, or through clinical excretions (lachrymal, nasal, saliva and faeces). PPRV is temperature labile and readily inactivated outside its host in a dry environment (Rossiter and Taylor, 1994). Infected and recovered animals develop life-long protective immunity with no carrier state (Hamdy *et al.*, 1976). However, virus can spread in animals as a mild virulent form that can later lead to severe disease where transmission occurs to naive susceptible populations of small ruminants (Couacy-Hymann *et al.*, 2007a).Other host factors like age, sex, breed and season may also play a role in disease outbreaks. Sheep and goats are the natural host for PPRV. More frequent outbreaks have been reported in goats than in sheep populations suggests that goats are more susceptible than sheep to an acute form of the disease (Lefevre and Diallo, 1990). The greater susceptibility to infection seen in goat breeds needs to be further investigated.

Table 1.5 Reported PPR infections in wildlife species

Common name	Latin name	Mode of detection	Country	Reference
White-tailed deer	*Odocoileus virginianus*	Clinical	USA (experimental infection)	(Hamdy and Dardiri, 1976)
Laristan sheep	*Ovis gmelini*	Clinical	Al Ain , Arabian Gulf	(Furley *et al.*, 1987)
Gemsbok	*Oryx gazella*	Clinical	Al Ain , Arabian Gulf	(Furley *et al.*, 1987)
Dorcas gazelles	*Gazella dorcas*	Clinical	Al Ain , Arabian Gulf	(Furley *et al.*, 1987)
Nubian Ibex	*Capra nubiana*	Clinical	Al Ain , Arabian Gulf	(Furley *et al.*, 1987)
African Grey dukier	*Sylvicapra grimma*	Serology	Nigeria	(Ogunsanmi *et al.*, 2003)
Thompson's gazelle	*Eudorcas thomsonii*	Clinical	Saudi Arabia	(Al-Afaleq *et al.*, 2004)
Arabian oryx	*Oryx leukoryx*	Serology	Saudi Arabia and UAE	(Frolich *et al.*, 2005)
Bubal hartebeests	*Alcelaphus buselaphus*	Serology	Ivory Coast	(Couacy-Hymann *et al.*, 2005)
Buffaloes	*Syncerus caffer*	Serology	Ivory Coast	(Couacy-Hymann *et al.*, 2005)
Defassa waterbuck	*Kobus defassa*	Serology	Ivory Coast	(Couacy-Hymann *et al.*, 2005)
Kobs	*Kobus kob*	Serology	Ivory Coast	(Couacy-Hymann *et al.*, 2005)
Arabian mountain gazelles	*Gazella gazella cora*	Clinical, serology and genome	UAE	(Kinne *et al.*, 2010)
Springbuck	*Antidorcas marsupialis*	Clinical, serology and genome	UAE	(Kinne *et al.*, 2010)
Arabian gazelles	*Gazella gazelle*	Clinical, serology and genome	UAE	(Kinne *et al.*, 2010)
Barbary sheep	*Ammotragus lervia*	Clinical, serology and genome	UAE	(Kinne *et al.*, 2010)
Bushbucks	*Tragelaphus scriptus kinne*	Clinical, serology and genome	UAE	(Kinne *et al.*, 2010)
Impala	*Aepyceros melampus*	Clinical, serology and genome	UAE	(Kinne *et al.*, 2010)
Rheem gazelles	*Gazella subguttorosa marica*	Clinical, serology and genome	UAE	(Kinne *et al.*, 2010)
Afghan Markhor goat	*Capra falconeri*	Clinical, serology and genome	UAE	(Kinne *et al.*, 2010)
Persian gazelle	*Gazella subgutturosa*	Serology	Turkey	(Albayrak and Gur, 2010)
Bharal	*Pseudois nayaur*	Clinical, serology and genome	China	(Bao *et al.*, 2011)
Sindh Ibex	*Capra aegagrus blythi*	Clinical, and serology	Pakistan	(Abubakar *et al.*, 2011)
Wild goat	*Capra aegagrus*	Clinical and serology	Kurdistan	(Hoffmann *et al.*, 2012)

1.6 Disease diagnosis

PPR can be confused with other diseases such as rinderpest, bluetongue and contagious caprine pleuropneumonia (CCPP) due to similarities in clinical signs. PPR identification also becomes complicated by secondary infection specifically due to pasteurella infection. Therefore, in addition to clinical observations in the field, PPR requires a laboratory based confirmatory test. The laboratory tests currently available for PPR diagnosis are grouped into three categories; i) those that detect antigen (virus isolation, antigen capture ELISA, lateral flow devices), ii) those that detect genetic material (RT-PCR, real-time PCR, LAMP PCR); and iii) those that detect antibodies (virus neutralisation test [VNT], competitive ELISA and indirect ELISAs). Laboratory test methods for the diagnosis of PPR that are recommended by OIE in the manual of diagnostic tests and vaccines for terrestrial animals are listed in Table 1.6. Regardless of the test applied, the efficiency of laboratory diagnosis is greatly influenced by the integrity of the sample received, with a number of factors affecting this during collection and transportation.

The VNT is the OIE gold standard test for PPR diagnosis and is a time-consuming method. Competitive ELISA based on H and N proteins of PPRV for detection of antibodies and RT-PCR including real-time RT-PCR, are generally used in PPR diagnosis. Recently, immunochromatographic lateral flow devices have been developed as a pen-side test using a monoclonal antibody (mAb) C77, specific to the PPRV H protein (Bruning-Richardson *et al.*, 2011) and validated in field conditions that could detect cases as early as 4 dpi, before onset of severe clinical signs (Baron *et al.*, 2014b).

Table 1.6 OIE listed laboratory test methods available for the diagnosis of PPR

Method	Purpose					Reference	
	Population freedom from infection	Individual animal freedom from infection	Confirmation of clinical cases	Prevalence of infection-surveillance	Immune status in individual animals or populations post-vaccination		
Competitive ELISA	++	++	-	+++	+++	c-H ELISA	(Anderson and McKay, 1994; Saliki *et al.*, 1993)
						c-N ELISA	(Libeau *et al.*, 1995)
Virus neutralisation	+++	+++	-	+++	+++		
PCR	-	-	+++	-	-	RT-PCR based on F gene	(Forsyth and Barrett, 1995)
						RT-PCR based on N gene	(Couacy-Hymann *et al.*, 2002)
						Multiplex RT-PCR	(Balamurugan *et al.*, 2006; George *et al.*, 2006)
						LAMP	(Li *et al.*, 2010)
						Real-time RT-PCR (qRT-PCR)	(Bao *et al.*, 2008; Batten *et al.*, 2011; Kwiatek *et al.*, 2010)
Virus isolation in cell culture	-	-	++	-	-	Cells-Vero, B95a and the Vero expressing dog SLAM, CV1 expressing goat SLAM	(Adombi *et al.*, 2011)
Immuno-capture ELISA	-	-	+++	-	-		(Libeau *et al.*, 1994)
Agar gel immune-diffusion	-	-	+	-	+		(Durojaiye, 1987)
Counter immune-electrophoresis	-	-	+	-	-		(Majiyagbe *et al.*, 1984)

+++, recommended method; ++, suitable method; +, may be used in some situations, but cost, reliability, or other factors severely limits its application; -, not appropriate for the purpose. The table adapted from OIE manual (2012) by including references.

1.7 Immunology

The immune response to morbillivirus infection involves both the innate and adaptive host immune system. Due to the lymphotropic nature of morbilliviruses, a profound immunosuppression is often seen following virus infection that allows opportunistic secondary infections to develop. The humoral immune response has been clearly defined following morbillivirus infection and more recent reports have detailed the role of cell-mediated immunity in response to infection. Host innate immune responses to virus infection are induced by the detection of pathogen-associated molecular patterns (PAMPs) by cellular pattern recognition receptors (PRRs). A complex network of intracellular signalling pathways are activated and resulting in transcription of host defence genes, predominantly pro-inflammatory cytokines including IFNs. Several classes of viral PAMP have been identified and include dsRNA, uncapped ssRNA and viral proteins (Akira *et al.*, 2006). The PRRs include toll-like receptor (TLRs) located at the plasma membrane and endosomal compartments. The PRRs located within the cytoplasm of infected cells include the retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) family, melanoma differentiation-associated protein 5 (MDA5) and dsRNA-dependent protein kinase R (PKR). The IFNs elicit distinct antiviral effects and are grouped into three types (I, II and III) according to their amino acid sequence (Randall and Goodbourn, 2008).

PPRV is highly lymphotropic, a factor that may leads to severe immunosuppression as described for other morbilliviruses (Pope *et al.*, 2013; Rajak *et al.*, 2005b). Virulent PPRV isolates cause marked immunosuppression, whereas the PPR vaccine induces only transient leukopenia without significantly affecting the immune

response (Rajak *et al.*, 2005b). Due to the highly lymphotrophic nature of morbilliviruses, dendritic cells (DCs) and/or macrophages are the immune cells most commonly targeted by morbillivirus infection. DCs play a role in the transport of virus to bronchus-associated lymphoid tissue or local lymph nodes where the virus is amplified and subsequently disseminated following an increase in viral load that leads to viremia (Lemon *et al.*, 2011). DCs express RIG-I and MDA5, which recognise virus and induce type I IFN production (Servet-Delprat *et al.*, 2003). Paramyxovirus accessory proteins are involved in limiting IFN production by interfering with induction and signalling pathways. The paramyxovirus C protein has been shown to inhibit the synthesis of viral mRNA and dsRNA interacting with the L protein during the formation of polymerase complex (Curran *et al.*, 1992), thereby preventing PKR mediated shut down of host cell protein synthesis (Gainey *et al.*, 2008). The paramyxovirus V protein has been found to interact with MDA-5 to block IFN induction pathway (Childs *et al.*, 2007; Goodbourn and Randall, 2009). Morbilliviruses have adapted distinct mechanisms to block the IFN signalling pathways, reportedly targeting multiple components. All the morbillivirus V proteins can block type I IFN action and to varying abilities can block type II IFN action (Chinnakannan *et al.*, 2013). The V protein of PPRV has been shown to inhibit the phosphorylation of STAT-1, STAT-2 and interferon-receptor-associated kinase Tyk2 that are involved in IFN signalling pathways (Chinnakannan *et al.*, 2013). Studies with the V protein of RPV showed that the N-terminal domain interacts with STAT-1, whilst the C-terminal V-specific domain interacts with the IFN receptor associated kinase Jak1 and Tyk2 (Chinnakannan *et al.*, 2014). A recombinant MV knockout for V protein that was unable to antagonise STAT-1 function and found to be attenuated *in vivo* (Devaux *et al.*, 2011).

Virus transfer to T cells is also mediated by the DCs and leads to the induction of T cell silencing mechanisms. The H and F proteins of MV have been shown to interfere with the proliferation of T cells, causing cell cycle arrest by interfering with progression in the S-phase of the cell cycle without inducing apoptosis (Avota *et al.*, 2010; Engelking *et al.*, 1999; Schlender *et al.*, 1996). Blood from PPRV infected goats was analysed for specific immune cell sub-set count and it has been shown that no change in the proportion of $WC1^+$ γ/δ T cells and $CD14^+$ monocyte/ macrophage cells is seen. However, the $CD4^+$ cells were found to decrease from 4 dpi and the $CD8^+$ cells remained unchanged during the initial days and by 7 dpi the proportion of $CD8^+$ had slightly increased, although the exact reason for this observation remains undefined (Herbert *et al.*, 2014).

The immunosuppressive effect of PPRV infection can have severe outcomes in the field depending on the nutritional status of the host and the presence of existing infections. In natural infection, immunocompromised animals are prone to secondary infections. Leukopenia is often observed from 4 dpi although depending on numerous factors the overall outcome of infection may not be sever and recovery may occur (Baron *et al.*, 2014a; Herbert *et al.*, 2014; Pope *et al.*, 2013).

From a vaccination perspective, the presence of maternal antibodies that neutralise PPRV have been detected in young animals for up to 3-4 months after birth (Libeau *et al.*, 1992). Therefore, vaccination in new-borns is not necessary until 3-4 months of age (Bodjo *et al.*, 2006). Animals vaccinated with attenuated strains or animals infected and recovered will develop protective immunity for life (Boer *et al.*, 1975;

Diallo *et al.*, 2007). Cell-mediated and humoral immune responses against PPRV are mainly directed against the H, F and N proteins (Sinnathamby *et al.*, 2001). However, immunisation with PPRV glycoproteins (H and/or F) induces protective humoral immunity whilst anti-N antibodies are not able to neutralise virus (Diallo, 2003; Sinnathamby *et al.*, 2001). Interestingly, both B cell and T cell epitopes have been mapped to the N protein of PPRV (Choi *et al.*, 2005; Mitra-Kaushik *et al.*, 2001) and a further study verified a B cell epitope at the C-terminus of the N protein (Dechamma *et al.*, 2006). A B-cell epitope has also been mapped to the H protein of PPRV (Renukaradhya *et al.*, 2002). The $CD8^+$ T and not $CD4^+$ cells were shown to be primed upon challenge with virulent PPRV in animals vaccinated with PPRV H protein (Herbert *et al.*, 2014).

1.8 Disease control

Preventive measures employed in an uninfected area include the restriction of animal importation from disease-infected regions. Disease can be efficiently controlled by the isolation and slaughtering of infected animals, disinfection of the environment and the quarantine of infected animals. Prophylactic immunisation in suspected animal populations or areas is also recommended. Immunisation is carried out with the availability of excellent live attenuated vaccines that elicit protective immunity that is maintained for at least three years (Diallo *et al.*, 2007). These vaccines are thermolabile and require maintenance of a cold storage chain in the hot tropical climate where PPR is endemic. The major obstacle to PPRV control is the requirement for frequent immunizations, at least every 3 years, due to the high

turnover of small ruminant populations. Vaccination in younger animals of 4 to 6 months old is recommended (Balamurugan *et al.*, 2012b).

1.8.1 Live attenuated PPRV vaccine

For many years, in the absence of a homologous vaccine, the 'Tissue Culture Rinderpest Vaccine' (TCRV) was used as a heterologous PPR vaccine. Its success was based on the cross reactivity of neutralising antibodies to the disease (Mariner *et al.*, 1993). However the use of the TCRV was prohibited in 1996 by the Food and Agriculture Organization (FAO) to enable post-eradication sero-surveillance of any remaining circulating RPV. To attempt PPRV vaccine isolation, PPRV was initially grown on primary cultures of sheep liver cells (Gilbert and Monnier, 1962). Serial passage of PPRV in primary cell culture for attenuation was unsuccessful even after 65 passages (Benazet, 1984). Later in 1989, Diallo et al. successfully obtained a highly attenuated PPRV isolate (Nigeria 75/1) following serial passage in Vero cells for 63 passages. The virus was found to have lost its pathogenicity and elicited protective immunity (Adu *et al.*, 1990; Diallo *et al.*, 1989). Similarly, at least three more vaccine strains Sungri 96, Arasur 87 and Coimbatore 97, were developed in India by 75 serial passages of PPRV in Vero cells (Sen *et al.*, 2010; Singh *et al.*, 2010). Currently, Nigeria 75/1 (lineage II) and Sungri 96 (Lineage IV) are widely used in endemic areas for the vaccination of small ruminant populations. whereas Arasur 87 is only used regionally in India. Details of these three vaccines are provided in Table 1.7. Following further development the PPRV live attenuated vaccines were demonstrated to be potent, safe and efficient in inducing a protective immunity that is maintained for at least three years in goats and sheep following

inoculation of a single dose of vaccine (Diallo *et al.*, 2007; Saravanan *et al.*, 2010). The PPRV Nigeria 75/1 vaccine has also been found to protect against challenge by virulent virus by providing sterile immunity and thus prevents further spread of disease (Couacy-Hymann *et al.*, 1995). These vaccines are manufactured in freeze-dried form with various chemical stabilizers to make a thermostable product (Mariner *et al.*, 1993; Worrall *et al.*, 2000), a critical factor in Africa and Asia.

Table 1.7 Characteristics of the live attenuated PPR vaccines.

Parameters	PPRV Nigeria 75/1	PPRV Sungri 96	PPRV Arasur 87
Passage and origin	LK-6, BK-2, Vero-63 Nigeria. sheep	B95a-10, Vero-59 North India, Goat	Vero 75 South India, Sheep
Complete CPE	3-6 days	3-6 days	2-3 days, rapid growing
Safety in pregnancy	Safe in pregnancy		
Lineage	II	IV	IV
Usage	Extensively used in several countries	Extensively used in India (> 20million doses)	Used in some states of India
Virus sequence	Full genome sequenced	Full genome sequenced	Not available

1.8.2 Recombinant subunit vaccines

Initial work carried out with a bivalent capripox virus (CPV) vaccine expressing the F and H proteins of RPV has been shown to protect goats against PPRV (Romero *et al.*, 1995). CPV expressing the homologous PPRV proteins H (Diallo *et al.*, 2002b) or F (Berhe *et al.*, 2003b) were also shown to protect goats against PPRV infection and later this was confirmed in both goats and sheep (Chen *et al.*, 2010). The concern about the effectiveness of CPV expressing PPRV F and H vaccine in sheep and goats with pre-existing immunity against either of the capripox or PPR viruses was addressed recently (Caufour *et al.*, 2014). In this experiment, animals were

immunised with either with a capripox or a conventional PPR vaccine. These animals with pre-existing immunity were inoculated with a recombinant bivalent CPV expressing the PPRV F and H glycoproteins. After four weeks of immunisation with CPV expressing the F and H proteins, the animals were challenged with a virulent CPV strain followed by virulent PPRV three weeks later. Complete protection against CPV challenge in animals pre-immunised either with PPRV or the CPV vaccine was observed. Unfortunately, only partial protection was obtained against PPRV challenge in animals pre-immunised with the CPV expressing PPRV glycoproteins. This suggested a limited replication of CPV expressing PPRV F and H proteins in the presence of pre-existing antibodies against CPV. The low replication led to the poor expression of PPRV F and H proteins and eventually low level of antibody response produced against PPRV F and H proteins.

A further subunit vaccine, a vaccinia virus vector (modified vaccinia Ankara) expressing PPRV F and H proteins, was also shown to protect goats against PPRV infection (Chandran *et al.*, 2010). However, this vaccine required two doses to stimulate sufficient neutralising antibodies to protect against PPR, a regimen that is not feasible in a PPR disease control programme due to the increase in cost with the requirement for multiple vaccinations. Goats immunised with recombinant fowl pox (rFP) expressing H or F proteins of PPRV were shown to induce poor antibody responses (Herbert *et al.*, 2014). Recombinant adenovirus vectors expressing PPRV glycoproteins were developed recently (Herbert *et al.*, 2014; Qin *et al.*, 2012; Rojas *et al.*, 2014; Wang *et al.*, 2013a). A recombinant replication-defective human adenoviruses serotype 5 incorporating H or F proteins of PPRV was shown to protect

goats (Herbert *et al.*, 2014) and sheep (Rojas *et al.*, 2014) against challenge with virulent PPRV and induced both humoral and cell mediated immunity.

Subunit vaccines developed based on a baculovirus expression system producing H (Sinnathamby *et al.*, 2001) and F proteins (Rahman *et al.*, 2003) or the H protein expressed in transgenic peanut plants (Khandelwal *et al.*, 2011) need to be assessed further for safety and vaccine potency. Similar assessments are lacking for the recently developed suicidal DNA vaccine (based on the Semliki forest virus replicon-based expression systems) utilizing the H gene of PPRV (Wang *et al.*, 2013b) and a virus like particle (VLP) preparation composed of M and H or N proteins (Liu *et al.*, 2014a; Liu *et al.*, 2014b).

1.8.3 Reverse genetics techniques to make DIVA and multivalent vaccines

Reverse genetics is defined as a method in which a live virus is recovered in cell culture via a cDNA copy of the RNA genome. In other words, it provides a means to manipulate RNA virus genomes through cDNA copies of the RNA genome. This technique has been utilised extensively to further the basic understanding of virus gene function, virus-cell interaction, and pathogenesis.

The establishment of these techniques for positive strand RNA viruses was rapid as it was quickly shown that their genomes acted as mRNA. To recover positive strand virus, the cDNA plasmids of virus genome or the RNA transcripts obtained from those plasmids are transfected into susceptible cells to recover the virus. Poliovirus was the first positive strand RNA virus to be rescued from a cDNA genome plasmid

(Racaniello and Baltimore, 1981) and the rescue of other positive stranded RNA viruses quickly followed.

Establishment of reverse genetics system for negative stranded RNA viruses took longer to develop due to several biological challenges identified when working with them. Primarily, the genomes and anti-genomes of negative stranded RNA viruses cannot function as mRNA. The realisation that the genome and anti-genome RNA must be entirely encapsidated with N protein to form an RNPs that acts as the substrate for the RdRP was a breakthrough in our understanding of these viruses. To enable virus rescue, a full length genome plasmid cDNA is co-transfected with helper plasmids encoding the proteins necessary for the formation of RNPs to initiate virus replication. The genome and helper plasmids are often cloned under the transcriptional control of a T7 polymerase promoter. The genome and helper plasmids are then transfected into a suitable cell line that contain T7 polymerase, supplied by either a rFP expressing T7 or through endogenous expression of T7 polymerase within the cell line used. A further realisation was that the primary transcript produced from the full-length genome plasmid should be of positive sense (antigenome) otherwise the mRNA generated from the helper plasmids will hybridize with negative sense genome transcripts, inhibiting the RNP complex formation.

Rabies virus was the first negative-strand RNA virus recovered completely from cDNA in 1994 (Schnell *et al.*, 1994). The approach was soon adapted to rescue paramyxoviruses including MV (Radecke *et al.*, 1995), RPV (Baron and Barrett, 1997), Newcastle disease virus (NDV) (Peeters *et al.*, 1999), CDV (Gassen *et al.*,

2000), Mumps (Clarke *et al.*, 2000), respiratory syncytial (Collins and Murphy, 2005), Nipah (Yoneda *et al.*, 2006) and Hendra (Marsh *et al.*, 2013) viruses. A reverse genetics system for RPV, a closely related morbillivirus to PPRV, was first established in 1997 (Baron and Barrett, 1997) by transfecting the full-length cDNA of RPV and helper plasmids (N, P and L) of RPV into B95a cells that were pre-infected with a recombinant vaccinia virus expressing T7 RNA polymerase (Figure 1.7). Further, this RPV reverse genetics system with some modifications (using Vero cells and rFP expressing T7 polymerase) was used to develop a chimeric RPV-PPRV (F/H) virus from a modified cDNA clone. The modified RPV-PPRV (F/H) clone was constructed by replacing the F and H genes of RPV with the heterologous PPRV F and H genes. However the rescued chimeric virus was severely debilitated in cell culture achieving only very low titres (Das *et al.*, 2000). To increase the titre of the recombinant virus it was necessary to include the homologous F and H proteins alongside the homologous M protein from PPRV (Mahapatra *et al.*, 2006). The new RP-PPRV (M/F/H) rescued chimeric virus grew efficiently in cell culture and protected goats from challenge with virulent PPRV (Mahapatra *et al.*, 2006). Similarly, the N gene of PPRV was swapped into the RPV cDNA clone with the objective of developing a marker vaccine for RPV (Parida *et al.*, 2007). This chimeric virus protected cattle challenged with virulent RPV. Further, using this chimeric virus serological differentiation of vaccinated animals from non-vaccinated infected animals was possible using a newly developed ELISA based on variable part of RPV N gene (Parida *et al.*, 2007). However, this RPV-PPRV (N) chimeric virus was not utilised in field conditions during the final stages of eradication. To attempt to develop a positive marker vaccine for RPV, the GFP gene was inserted as a separate transcriptional unit in the RPV cDNA clone and was rescued (Walsh *et*

al., 2000a; Walsh *et al.*, 2000b). Using this established reverse genetics method a recombinant RPV was rescued that lack C and V proteins of RPV that helped to reduce the pathogenicity of the virus (Baron and Barrett, 2000). Similarly, the promoter regions of RPV were swapped between vaccine and wild type strain of the RP viruses to determine the pathogenicity in cattle (Banyard *et al.*, 2005).

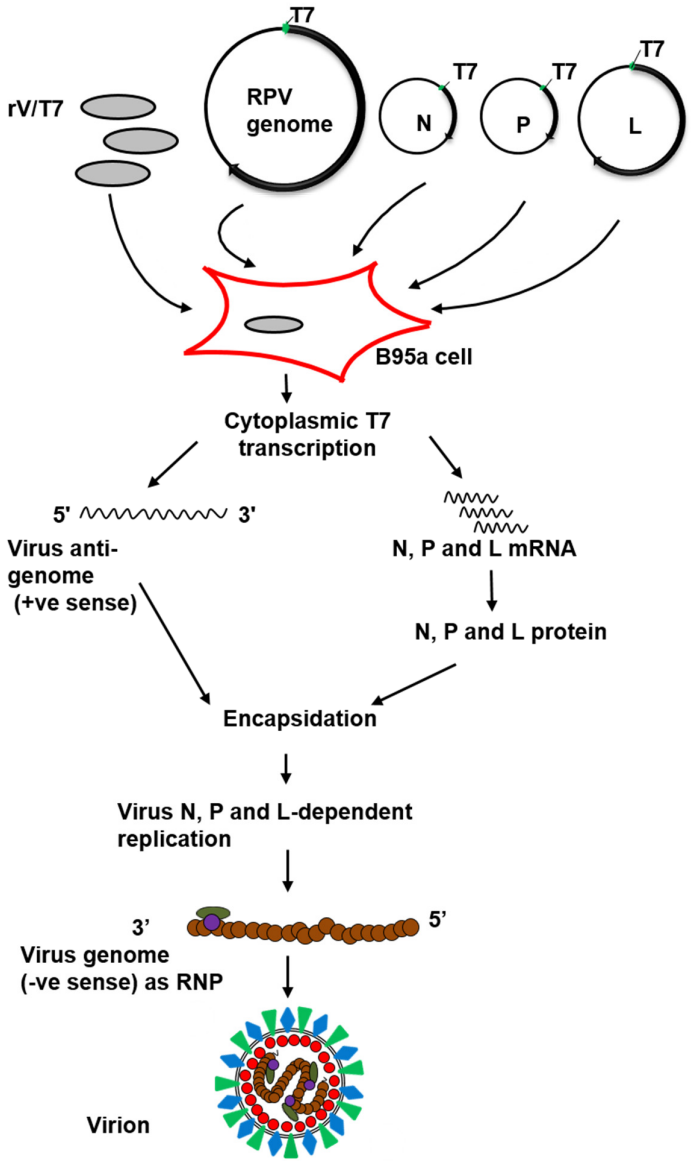

Figure 1.7 Schematic representation of the reverse genetics method to generate infectious recombinant RPV. The full-length genome (cDNA) plasmid and the helper plasmids (N, P and L) were constructed under T7 polymerase promoter. The B95a cells were infected with recombinant vaccinia expressing T7 polymerase (rV/T7). The genome and helper plasmids were transfected into B95a cells and were transcribed by the T7 polymerase. The N, P and L proteins bound to the viral RNA anti-genome strand to form the ribonucleocapsid protein (RNP) complex, a minimum necessary component required to initiate the replication cycle and produce complete virus.

Establishment of reverse genetics system for PPRV was unsuccessful despite several attempts (Bailey, 2006), although the PPRV mini-genome rescue system was successfully developed (Bailey *et al.*, 2007). The reason for being unable to rescue PPRV from a full-length PPRV cDNA described were proposed to be associated with sequence errors in the final full length cDNA, specifically the presence of potentially lethal mutations in the UTR at the M and F gene junction (Bailey, 2006). Therefore the main aim of this PhD project was to establish a reverse genetics system for PPRV after re-sequencing and confirming the correct sequence in the full length genome of PPRV.

Vaccination of small ruminants with the current live attenuated PPRV vaccine strains and natural virus infection induces an antibody profile that is indistinguishable. Because of this, the development of a marker vaccine with companion diagnostic tests is required to fulfil the 'DIVA' strategy and enable the differentiation between naturally infected and vaccinated animals within a population. The availability of a DIVA vaccine would greatly enhance serological surveillance during any eradication initiative for PPRV in future (Baron *et al.*, 2011; Buczkowski *et al.*, 2014).

Existing DIVA strategies for PPRV have focussed on developing subunit vaccines expressing the PPRV F and/or H gene in viral vectors such as poxviruses (vaccinia, Capripox and fowl pox) and adenoviruses (canine or human types) as explained in Section 1.8.2. The absence of the PPRV N protein in these subunit vaccine preparations facilitates serological identification of infected animals using an ELISA based on the N protein. However, such subunit vaccines often require multiple doses

and may have reduced efficacy due to potential pre-existing immunity to the viral vector, reduced antibody induction through an inability to replicate, the potential for short lived antibody responses and/or potentially high costs of recombinant vaccine production. An alternative strategy is to use the existing live attenuated PPRV vaccine by manipulating in a specific region or epitope of a viral protein sequence of a cDNA clone using reverse genetics system to obtain positively and or negatively marked vaccines. Therefore this PhD study aimed to rescue the widely used vaccine strain (Nigeria 75/1) from a cDNA clone. To make it a positive marker vaccine, similar to RPV, an extraneous transcriptional unit of GFP was inserted and expressed. Alongside this, attempts have been made to negatively mark the vaccine by creating a recombinant that is epitope deleted for part of the anti-PPRV H C77 monoclonal antibody (mAb) binding site, a key component of the current diagnostic competitive H ELISA (c-H ELISA). The C77 mAb and the antibody present in the test sera compete with each other to bind to the specific epitope on the H protein of coated antigen. The assay works on the principle that antibodies to PPRV in the test sera can block the binding of the mAb to the antigen. The C77 mAb is detected using rabbit anti-mouse IgG conjugated to horseradish peroxidase. Therefore, it is hypothesised that the negatively marked vaccine virus with a mutated C77 mAb binding epitope would facilitate the c-H ELISA to distinguish serologically vaccinated animals from those naturally infected with circulating viruses. Antibodies in naturally infected animals could be detected using c-H ELISA whereas the protected vaccinated animals will not be detected.

As many diseases in small-ruminants overlap geographically with PPRV (e.g. CPV, bluetongue virus [BTV], Rift valley fever virus [RVFV], contagious ecthyma and border disease virus) (Malik *et al.*, 2011; Mondal *et al.*, 2009; Ozmen *et al.*, 2009;

Saravanan *et al.*, 2007; Toplu *et al.*, 2012) the idea of increased valency was investigated using a PPRV vaccine as a vehicle for the expression of heterologous proteins. A single vaccination programme covering multiple diseases would greatly improve the efficiency of control programmes by increasing productivity of human resources through the reduction in the overall cost of vaccine administration. Therefore, the final aim of this PhD study was to make a multivalent vaccine using PPRV as a viral vector. As the PPRV live attenuated vaccines provide lifelong immunity in small ruminants (Diallo *et al.*, 2007; Hamdy *et al.*, 1975; Sen *et al.*, 2010), using reverse genetics technique immunogens from co-circulating viruses (stated above) may be inserted into PPRV genome to make a multivalent vaccine. However, insertion of multiple larger insertions as separate transcriptional units in full length genome of virus could reduce growth of recombinant virus due to effects on the transcriptional gradient. To overcome this polar attenuation recombinant MV (Takeda *et al.*, 2006) and NDV (Gao *et al.*, 2008) with genomes divided into two or three segments were generated using reverse genetics systems. These tolerated multiple larger insertions as separate transcriptional units. A two-segment MV genome was obtained by constructing the first plasmid containing N, P, M and F genes and second plasmid containing H and L genes. Each gene cassette was flanking with the GP and AGP. The transcriptional gradient of expression of genes in the segmented virus was not hampered due to a reduction in the total length in comparison to the non-segmented genome. The segmented MV and NDV were shown to be efficient vectors expressing long and multiple (up to six) transgenes (Gao *et al.*, 2008; Takeda *et al.*, 2006). The coding capacity in segmented NDV was extended by 30%. A similar strategy could be applied to PPRV in developing multivalent vaccines for regionally relevant diseases.

1.9 Aims of the project

In the context of the background literature, the main objectives of this PhD studies were:

1. To re-sequence the whole genome of PPRV Nigeria 75/1 vaccine strain to confirm the correctness of existing sequences needed for cDNA synthesis and to eradicate any sequence error identified in the Turkey 2000 PPRV sequence determined previously.
2. To establish the reverse genetics technique to rescue PPRV Nigeria75/1 vaccine strain.
3. To investigate both positive and negatively marked forms of the vaccine as potential DIVA vaccines.
4. To attempt to increase vaccine valency through inclusion of a heterologous gene in the PPRV genome in either segmented or non-segmented forms.

Chapter 2 Materials and methods

2.1 Cells and viruses

2.1.1 Cells lines

Vero.DogSLAMtag (VDS) cells were used in this study. The Vero cell lineage (ATCC CCL-81) is an epithelial cell that was isolated from the kidney of the African green monkey (*Cercopithecus aethiops*). VDS cells are the Vero cells constitutively expressing the canine morbillivirus receptor SLAM under the selection pressure of zeocin (Seki *et al.*, 2003).

2.1.2 Culturing and maintenance of the cell lines

The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) foetal calf serum (FCS, Gibco), penicillin (100 U/ml, Sigma) and streptomycin sulphate (100 µg/ml, Sigma) at 37°C/ 5% CO_2. Tissue culture flask (175 cm^2) with confluent monolayer of cells were washed with 15 ml of calcium and magnesium (Ca/Mg) free phosphate buffered saline (PBS, Gibco) and overlaid with 5 ml of trypsin (0.25% solution, Gibco), incubated at 37°C for 5 minutes. DMEM containing FCS (15 ml) was added to the detached cells to stop further trypsinisation and centrifuged at 290 g for 5 minutes. The cell pellet was re-suspended in DMEM and the cells counted using a haemocytometer and passaged in a new tissue culture flask/ plate.

2.1.3 Virus stocks

PPRV Nigeria 75/1 recombinant viruses were generated through reverse genetics in this study and its parental virus, Nigeria 75/1 vaccine strain, was obtained from the OIE-FAO Reference Laboratory for PPR at The Pirbright Institute, Pirbright, UK. PPRV was grown on VDS cells to prepare high titres of virus stock. VDS cells of ~ 70% confluency were infected with virus at an multiplicity of infection (MOI) of 0.1 and incubated at 37°C/ 5% CO_2 for few days (usually 4-5 days) until the CPE and syncytia extended to 70-80% of total cell surface. Virus was harvested by one cycle of freezing and thawing followed by centrifugation at 290 g for 10 minutes to remove cell debris and the supernatant containing virus was stored at -80°C until further use.

2.1.4 Virus isolation

PPRV was isolated on VDS cells from the clinical samples. Cotton swabs were used to collect viral excretions in 1 ml of PBS. The liquid containing virus was used to infect the VDS cells in 25 cm^2 tissue culture flasks and incubated at 37°C for one hour with intermittent shaking rocking. The cells were washed once with 2 ml of PBS followed by addition of 5 ml of media (containing 2.5% FCS) and incubated at 37°C for a week. Cells were observed for the appearance of CPE and syncytia and were blindly passaged for at least two more times if no PPRV-specific CPE was observed.

2.1.5 Virus passage

The stable maintenance of introduced mutations or inserted gene expression in the recombinant viruses was assessed by serial passage in VDS cells. VDS cells of 70% confluent in 25 cm^2 tissue culture flask was infected with PPRV (at 1:200 dilution) and incubated at 37°C for few days until at least 80% CPE was observed. The virus was harvested by freeze-thawing once and clarified by centrifugation at 290 g for 10 minutes. The supernatant containing the viruses was used for successive passage.

2.1.6 Multi-step growth curves

VDS cells were plated at $2.5x10^5$ cells/ well in a six-well plate (BD falcon) and incubated overnight to reach ~70% confluency. Cell monolayers were washed once with DMEM and were infected (in duplicates) with 1 ml of virus inoculum diluted to 0.01 MOI in DMEM and incubated at 37°C for an hour with intermittent shaking for even distribution of the inoculum. The excess of the virus inoculum was removed and the cells were washed twice with DMEM. For each well 2 ml of DMEM containing 5% FCS was added and incubated at 37°C. The plates were frozen at 0, 12, 24, 36, 48, 60, 72, 84 and 96 hour post-infection (hpi). The virus was harvested by freeze-thawing and cell debris clarified by centrifuging at 290 g for 10 minutes. The supernatant containing the virus was stored at -80^o C until use.

2.1.7 Determination of virus titre

Virus titre was determined by 50% tissue culture infectious dose ($TCID_{50}$) in 96-well plates. Virus stock was serially diluted 10 fold outside the microtiter plate using serum free DMEM from neat, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-11}. Aliquots of 50 µl virus each was added into 8 wells in a 96 well plate, one

column for each dilution of virus, where the first column is for neat virus and last column is for cell control. VDS cell suspension of $5x10^4$/ml was prepared in DMEM. VDS cell suspensions of 100 µl was added to all the wells and incubated for 5 days at 37°C/ 5% CO_2. The CPE was observed under microscope and scored. The virus titre was calculated according to Read and Muench and expressed in log_{10} values (Reed and Muench, 1938). All assays using plaques have a lower limit of detection and the detection limit of this assay is more than 1 log_{10}.

2.1.8 Bacterial cells

The JM109 strain of *Escherichia coli* was used for the transformation and cloning of plasmid DNA. The bacterial cells were plated on Luria Bertani (LB) agar and incubated overnight at 37°C. A single colony of bacteria was picked and grown overnight in LB broth and used for the preparation of competent cells for transformation. Glycerol stocks of the bacteria (15%) prepared and stored at -80°C for future use.

2.2 RNA and DNA techniques

2.2.1 RNA extraction

Viral RNA was extracted either using robotic extraction methods (MagNA Pure LC Total Nucleic Acid Isolation Kit, Roche, UK) or Qiagen viral RNA mini kit. Robotic RNA extraction method was followed for a large number of animal experimental samples, to maintain uniformity by reducing bias in extraction method for the precise comparison of viral RNA amount between samples. Otherwise, in all other experiments Qiagen Viral RNA mini kit was routinely used following the

manufacturer's protocol. Briefly, 140 µl of virus infected cell supernatant was mixed with 560 µl of kit supplied lysis buffer and left at room temperature for 10 minutes. To this mixture 560 µl of absolute ethanol was added and vortexed. This solution was passed through QIAamp mini spin column by centrifuge at 8000 g for 1 minute. The flow through was discarded and column filter was washed with 500 µl of wash buffer-1 (spun at 8000 g for 1 minute) and 500 µl wash buffer-2 (spun at 8000 g for 3 minutes). The column was transferred to another clean and sterile tube and 50 µl of nuclease-free water was added and incubated for a minimum of one minute at room temperature. The column was centrifuged at 8000 g for 1 minute to elute RNA from the column and the RNA was stored at -80°C until further use.

2.2.2 Quantification of nucleic acids

The concentration and purity of DNA/ RNA was assessed by using spectrophotometer (NanoDrop 1000 Thermo Scientific) according to the manufacturer's protocol. Briefly, RNA or DNA samples (1.5 µl) were loaded on pedestal of the spectrophotometer and absorbance reading taken at 260 and 280 ηm. The reading at 260 ηm gives the concentration of nucleic acid in the sample and the ratio between the readings at 260 and 280 ηm estimates the purity of the DNA (1.8) and RNA (2.0).

2.2.3 Reverse-transcription polymerase chain reaction (RT-PCR)

The SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity polymerase kit (Life technologies) was used for reverse transcription of viral RNA into cDNA and further amplification in a single step. The RT-PCR mixture

contained 25 µl of 2x Reaction mix, 1 µl (10 pmol/µl) of each forward and reverse primer, approximately 50-100 ηg of template RNA and 1 µl (1 U) of SuperScript III RT/ Platinum Taq High Fidelity Enzyme Mix. The final reaction volume of 50 µl was made up with nuclease-free water and subjected to the following amplification cycles.

Step 1: Reverse transcription	50°C	30 minutes
Step 2: Initial denaturation	94°C	2 minutes
Step 3: Denaturation	94°C	15 seconds
Step 4: Primer annealing	55°C	30 seconds
Step 5: Elongation	72°C	1 minutes/Kb length of amplicon

Step 6: Steps 3 – 5 repeated for 36 cycles

Step 7: Final elongation	72°C	7 minutes
Step 8: Holding	4°C	

PCR products were stored at -20°C until further use.

2.2.4 Polymerase chain reaction

The PCR mix was prepared using KOD High-Fidelity PCR polymerase (Novagen) kit following the manufacturer's protocol. Briefly, reaction mix contain 5 µl of 10x buffer, 5 µl of dNTP mix (2 mM each), 3 µl of $MgS0_4$ (25 mM), 1.5 µl each of forward and reverse primers (10 pmol), 1 µl of KOD polymerase (1 U/µl), template DNA 50 to 100 ηg and final volume made up to 50 µl with water. The following amplification cycles was followed

Step 1:	Initial denaturation	94°C	2 minutes
Step 2:	Denaturation	94°C	15 seconds
Step 3:	Primer annealing	55°C	30 seconds
Step 4:	Elongation	72°C	15 seconds /Kb length of amplicon

Step 5: Steps 3 – 5 repeated for 36 cycles

Step 7:	Final elongation	72°C	7 minutes
Step 8:	Holding	4°C	

PCR products were stored at -20°C until further use.

2.2.5 Random amplification of cDNA ends (RACE)

The genome termini, leader and trailer sequence regions were amplified using 5' and 3' rapid amplification of cDNA ends as described by Li *et al*, and Tillett *et al* (Li *et al.*, 2005; Tillett *et al.*, 2000) using PPR virus specific primers (VSP). The primers sets used in RACE are provided in Appendix III. The Schematic diagram of steps involved in 5' and 3' RACE is shown in Figure 2.1. The cDNA synthesis was carried out using ThermoScript RT-PCR Systems (Life technologies) and RNA ligation using T4 RNA Ligase 1 (NEB). For 5' RACE, initially the cDNA was synthesised using virus specific primer P5-1 (Appendix III), template RNA digested by RNaseH treatment and the synthesised cDNA was ligated with adaptor (DT88). Adaptor constitutes modifications with '5'-phosphorylated, 3'-end inverted dA' to ensures ligation occurs between 5' end of adopter and 3' end of target DNA/RNA. For 3' RACE, first the adapter was ligated with viral RNA and followed by cDNA synthesis using DT89 primer that is unmodified and complementary to DT88 adopter. The cDNA obtained from both 5' and 3' RACE were further amplified

through primary and hemi-nested PCRs using virus specific primers (Appendix III). For primary PCR, DT89 was used with either P5-2 in 5' RACE or P3-1 in 3' RACE. For hemi-nested PCR DT89 was used with either P5-3 in 5' RACE or P3-2 in 3' RACE.

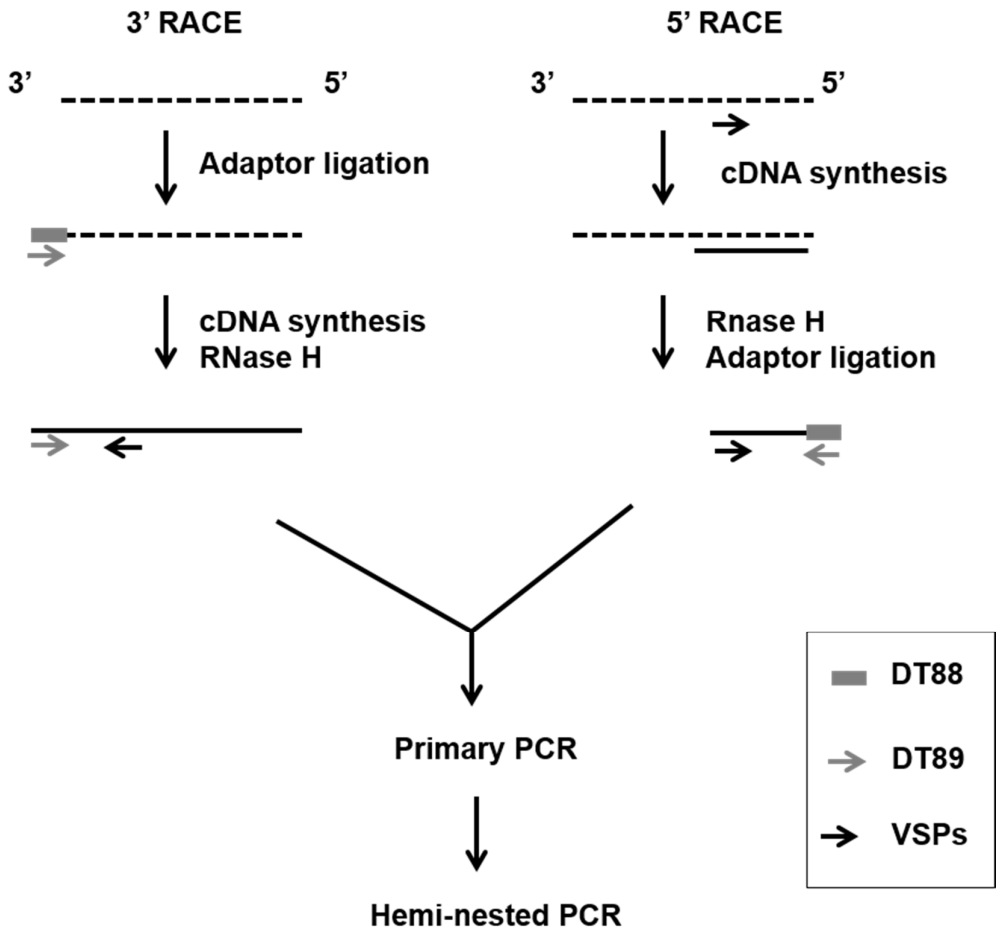

Figure 2.1 Schematic diagram depicting methods to establish the genome termini for PPRV. The dotted lines represent the RNA genome in 3' to 5' direction, the solid lines represent cDNA, DT88 is an adapter, DT89 primer is complementary to DT88 and the virus specific primers (VSPs). Figure taken from Li *et al.,* 2005.

2.2.6 Purification of DNA

DNA in PCR or restriction enzyme reactions was column purified using illustra GFX PCR DNA and Gel Band Purification Kit following the manufacturer's protocol and the final purified product was eluted in a final volume of 40 µl of DNase and RNase free water.

2.2.7 Agarose gel electrophoresis

Appropriate agarose gel (1 to 2 % based on DNA size) was prepared using electrophoresis grade agarose powder (Invitrogen) in Tris-acetate EDTA (TAE) buffer. GelRed (Biotium) was added to the molten agarose to obtain a final concentration of 1x and the gel was allowed to solidify for at least 30 minutes at room temperature. DNA samples were mixed with the sample loading dye (Invitrogen) before loading into the wells. 1Kb plus marker (Invitrogen) was used for the comparison of the DNA bands. Electrophoresis was performed using TAE buffer and at 100 V for an hour (5 V per centimetre). DNA bands were visualized on a gel documentation system (Biorad).

2.2.8 Extraction of DNA from agarose gels

The desired DNA fragment and non-specific or unwanted DNA present within a PCR or restriction enzyme digestion reaction was purified on agarose gel for use in the ligation or sequencing. Agarose gel (1%) was prepared using low melting point agarose powder (Invitrogen) and the electrophoresis was performed at ~ 20 V for 16 -20 hours for better separation of DNA fragments. The expected specific DNA fragment was excised using sterile scalpel and purified using Illustra GFX PCR

DNA and Gel Band Purification Kit (GE healthcare). The purified DNA fragments were quantified using Nanodrop 1000 spectrophotometer (Thermo Scientific).

2.2.9 Restriction enzyme digestion

Approximately 100-200 ηg of plasmid DNA was digested with an appropriate restriction enzyme(s) and 1x buffer (NEB) in a reaction volume of 10 µl and incubated at the recommended temperature for 2 hours. The digested product was analysed by electrophoresis. However, for the preparation of DNA fragments for ligation experiments, about 5 µg of plasmid DNA was digested with 5 U of enzyme in a reaction volume of 50 µl and incubated at the recommended temperatures overnight.

2.2.10 Phosphatase treatment

Antarctic phosphatase enzyme was used for the removal of 5´ phosphate group from the linear vector DNA fragments and, thus prevents the self-ligation during ligation reaction. It also decreases the vector background during cloning steps. Five µl of 10x Antarctic phosphatase buffer (NEB) and 1 µl (1 U/µl) of Antarctic phosphatase enzyme (NEB) was added to the restriction enzyme-digested vector and the mixture was incubated for 30 minutes at 37°C. The enzyme was heat inactivated at 70°C for 5 minutes followed by gel purification.

2.2.11 Ligation reactions

Ligation reactions in cloning was carried out using T4 DNA Ligase which catalyzes the joining of two strands of DNA between the 5´-phosphate and the 3´-hydroxyl

groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration. In a reaction volume of 10 µl, vector (30-40 ηg) and insert DNA at a molar ratio of 1:3 and 1 µl of 10x T4 DNA ligase buffer (Promega) and 1U of T4 DNA ligase enzyme (Promega) were added and incubated at 4°C overnight.

2.2.12 Preparation of competent cells and transformation

Competent *E. coli* JM109 cells were prepared fresh on the day of use by TSS-TCM method and transformed by ligation reaction or plasmid DNA as described below. An overnight culture of bacterial cells was diluted 1 in 200 in LB-broth medium and cultured at 37ºC until the OD_{600} reaches 0.25-0.35 (usually ~2 hours). The cells were centrifuged at 290 g for 10 minutes and re-suspended in $1/10^{th}$ volume of TSS (Appendix I) and left on ice for an hour. At the same time, the ligation reaction or plasmid DNA was diluted to 200 µl in TCM (Appendix I) and left on ice for an hour. The TCM DNA mix was added to ~ 200 µl of competent cells and left on ice for an hour. Heat shock treatment was given to the mixture at 42°C for 5 minutes and immediately cooled on ice for 5 minutes. SOC media (Appendix I) of 600 µl was added to the sample and incubated at 37ºC in a shaker for an hour. The cells were plated onto LB-agar plates containing selective antibiotics (ampicillin or kanamycin) and/or IPTG/X-gal and incubated overnight at 37ºC.

2.2.13 Plasmid mini and maxi preparations

Bacterial culture of approximately 1 ml (miniprep) or 500 ml (maxiprep) was used in plasmid DNA preparations. The Qiagen Miniprep Kit for purification of small scale (up to 20 µg) molecular biology grade plasmid DNA or the Qiagen Plasmid Maxi Kit

for purification of large scale (up to 500 µg) transfection-grade plasmid DNA were used following manufacturer's protocol. The kit procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica membrane in the presence of an appropriate salt concentration. The membrane columns are washed to remove impurities and the eluted DNA is concentrated and desalted by isopropanol precipitation.

2.2.14 DNA sequencing

BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems) based on Sanger sequencing was used where each of the four dideoxy terminators (ddNTPs) were tagged with different florescent dye. The cycle sequenced products were then cleaned to remove excess dye terminators and analysed through ABI-3730 automated sequencer.

The sequencing reaction mix was prepared as follows:

Primer (10 pmol/µl)	1 µl
BigDye reaction buffer (2x)	2 µl
BigDye enzyme mix	0.25 µl
DNA template (50-100 ηg)	1 µl
Nuclease-free water	5.75 µl

The cycle sequencing reaction was carried out using a thermal cycler under the following conditions with the initial denaturation of template DNA at 96°C for one minute.

Step 1. Denaturation at 96°C for 20 seconds

Step 2. Annealing at 50°C for 10 seconds

Step 3. Extension at 60°C for 4 minutes

The above steps were repeated for 30 cycles.

Removal of unincorporated dye terminators was carried out using EDTA/ethanol precipitation method. To the sequencing product 5 µl of 125 mM EDTA followed by 60 µl of absolute ethanol was added and the precipitated DNA was pelleted by centrifugation at 600 g for 30 minutes. The pellet was washed once with 60 µl of 70 % ethanol in water and vacuum dried. The pellet was re-suspended in 20 µl of Hi-Di Formamide (ABI) and loaded into a capillary and analysed through ABI-3730 automated sequencer.

2.2.15 Site-directed mutagenesis (SDM)

SDM was carried out to introduce the desired nucleotide changes in the plasmid DNA. Two complementary primers incorporating the nucleotide sequence changes intended in the target nucleotide sequence in the middle of the primers were designed. The denatured and circular single-stranded plasmid DNA acts as template during the PCR and the annealed mutagenesis primer were extended by polymerase incorporating the desired mutations in both the strands of the plasmid DNA.

The PCR mixture containing high fidelity enzyme KOD Hot Start DNA Polymerase (Novagen) was prepared as detailed below

10x KOD polymerase buffer (Novagen)	5 µl
25 mM Magnesium sulphate	3 µl
dNTPs (2 mM each)	5 µl
Sense primer	15 pM
Antisense primer	15 pM
Template DNA	~50 ηg
KOD Hot start DNA polymerase	1 µl (0.02 U/µl)

The thermal cycle reaction conditions were:

Step 1:	95°C for 2 minutes
Step 2:	95°C for 30 seconds
Step 3:	55°C for 30 seconds
Step 4:	70°C for 20 seconds per kbp of target amplification

Steps 2 to 4 were repeated for 25 cycles.

The resultant PCR product contained both the template plasmid DNA and the newly synthesized plasmid with introduced mutations. Since the template DNA was produced in JM109 strain of *E. coli*, it was DAM (DNA adenine methylase) methylated and therefore susceptible to *Dpn*I digestion unlike the newly synthesized plasmid DNA. The parental DNA (methylated) was digested by treating the PCR product with 1 µl (10 U/µl*) Dpn*I endonuclease (Promega) at 37 °C overnight. The *Dpn*I enzyme digested product (5 µl) was transformed into bacterial cells for further amplification and checked for the desired mutations by sequencing.

2.3 Data analysis

2.3.1 Nucleotide sequence analysis

The sequences generated by different PPRV specific primers were assembled using the Seqman program in DNAstar (DNAstar, USA). The assembled sequences were aligned using ClustalW implemented in BioEdit software v7.2.0 (Hall, 1999).

2.3.2 Statistical analysis

Statistical analysis was performed either in Microscoft Excel 2010 or GraphPad Prism v6.

Chapter 3 PPRV complete genome sequencing and analysis

3.1 Introduction

Following the successful eradication of RPV, PPR has been targeted by the OIE and FAO as the next viral pathogen to be eradicated (FAO, 2014). Currently, the disease is controlled by vaccinating small ruminants against disease using live attenuated vaccines. However, the inability of laboratory tests to distinguish between vaccinated and naturally infected animals means that serological surveillance following a mass vaccination campaign would be very inefficient, as seen during the RPV eradication programme. In order to understand the epidemiology of PPRV, sequence data are required. Currently only partial gene sequences exist for the majority of characterised PPRV isolates. To enhance our understanding of the variation of PPRV at the genome level we set about generating full genome data for PPRV isolates in our repository.

PPRV exists as a single serotype but at the genetic level four distinct lineages exist. This lineage differentiation is based on partial genome sequence of either N or F genes (Couacy-Hymann *et al.*, 2002; Forsyth and Barrett, 1995). Full genome data for PPRV is scarce with no full genome sequence for lineage III isolates being available and only seven complete genome sequences available representing the other lineages. At the start of this project only one lineage I isolate full genome was available, two lineage II genome sequences and four lineage IV genome sequences. Current control methods across PPRV endemic areas have often relied on the application of vaccine strains generated from heterologous PPRV lineages. For example, across West Africa, where lineage I and II have predominated and across

North Africa and China where lineage IV is present vaccination using the lineage II Nigeria 75/1 vaccine strain has been used. Therefore it is expected that PPR outbreaks caused by lineage III would be readily controlled by using heterologous lineage II (Nigeria 75/1) or lineage IV (Sungri/96) vaccines. However from a genetic standpoint it is important to determine the full genome sequence from circulating isolates. More importantly, studying the genetic evolution of PPRV enables an understanding of the epidemiology of the virus, knowledge that may impact on the use of vaccines to control the disease. Finally, the requirement for a DIVA vaccine requires that a suitable reverse genetics system for PPRV is available and as such we aimed to establish sequences from different isolates and vaccines strains to ensure that the genome sequence was correct, a pre-requisite for the generation of a functional reverse genetics system. As discussed in chapter 1, previous attempts to establish a reverse genetics system for PPRV were unsuccessful (Bailey, 2006). Although essentially undefined, potential reasons for this failure were hypothesised to include problems surrounding the sequence of the constructed full length clone. To this end, the first step in this study was to ascertain a complete correct genome sequence for the Nigeria 75/1 vaccine strain and compare it with the existing available sequence before ordering/ assembling a full-length cDNA clone. As a backup study, it was planned to rescue another widely used vaccine strain (Sungri/96) whose whole genome sequence was available without the genomic promoter region. Therefore, the sequencing of the whole genome sequence of the Sungri/96 strain was also planned. In a further attempt to generate useful epidemiological data, full genome sequencing of a lineage III PPRV isolate was also performed.

The molecular evolution of PPRV and its role in disease epidemiology has not been explored in detail. The current molecular epidemiology of PPRV, which is based on sequence comparison of a small region of the F gene (322 nt) or the N gene (255 nt), has identified 4 distinct lineages (I–IV) of PPRV (Banyard *et al.*, 2010). However, this analysis has not generated much information on the evolution and dispersal of each of the PPRV lineages. Many aspects of PPRV evolution, such as the origin of an ancestral virus, divergence and time of origin, and the historical and geographic patterns of spread, are poorly understood (Libeau *et al.*, 2014). A better understanding of the evolution of PPRV would enable the prediction of how these viruses will lead to further outbreaks and epidemics and provide data for control strategies including application of vaccines.

This Chapter describes the standardisation of PPRV complete genome sequencing techniques and the sequencing of both PPRV vaccine and field isolates. Further, a Bayesian analysis was carried out to study the evolutionary and epidemiological dynamics of PPRV using both complete and partial genome sequences of viruses from all the 4 lineages.

3.2 Materials and methods

3.2.1 PPR virus isolates

For complete genome sequencing of PPRV, seven isolates Nigeria 75/1, Sungri 1996, Uganda 2012, Ethiopia 2010, Morocco 2008, UAE 1986 and Oman 1983 were utilised in this study with the following passage history. PPRV Nigeria 75/1, UAE 1986 and Oman 1983 isolates stock were obtained from the OIE-FAO Reference

Laboratory for PPR at The Pirbright Institute, UK. PPRV Nigeria 75/1 is a live attenuated vaccine strain widely used in disease control (Diallo *et al.*, 1989). PPRV UAE 1986 virus was isolated from a *Dorcas gazelle* (Furley *et al.*, 1987). PPRV Oman 1983 virus was isolated from the caecal tissue of a goat from Ibri, Oman (Taylor *et al.*, 1990). PPRV Sungri 96, a commercially available vaccine strain generated by serial passage in cell culture (Sen *et al.*, 2010) was provided by Intervet International B.V, Boxmeer, The Netherlands. The PPRV Uganda 2012 isolate was derived from an infected goat, received as swab sample from Uganda. The PPRV Ethiopia 2010 virus was isolated from the intestinal suspension of an infected goat at National Veterinary Institute, Addis Ababa, Ethiopia. This PPRV Ethiopia 2010 isolate received as tissue suspension from Ethiopia. The PPRV Morocco 2008 isolate was derived from the mesenteric lymph node of an infected goat, following a single passage on VDS cells (Hammouchi *et al.*, 2012).

3.2.2 Complete genome sequencing of PPRV

Viral RNA was extracted from PPRV Nigeria 75/1, Sungri 1996, Uganda 2012, Ethiopia 2010, Morocco 2008, UAE 1986 and Oman 1983 isolates. Oligonucleotide primer sets designed based on highly conserved regions of PPRV genome sequences previously available in the GenBank (accession number NC006383, X74443, EU267273 and AY560591). All primer sequences including 46 forward and 32 reverse primers are provided in Appendix III. Seven to nine overlapping PCR fragments covering the entire PPRV genome were amplified using RT-PCR. The amplified PCR products were column purified and sequenced.

3.2.3 Sequence datasets

Six complete genome sequences were available in GenBank with the Sungri/96 sequence being complete with the exception of the GP. Seven complete genomes sequences of PPRV were generated in this study including re-sequencing of two vaccine strains (Nigeria 75/1 and Sungri 96) and an additional complete genome sequence (Ethiopia 1994 isolate) was obtained from the National Veterinary Institute, Sweden by Dr. M Munir (Table 3.1). However, of these 14 full genome sequences, Nigeria 1975/1 and Sungri 1996 represent vaccine strains generated after extensive serial passage of virus. Therefore, the evolutionary rate and time to most recent common ancestor (TMRCA) were compared with and without inclusion of the vaccine strains. The complete genome sequences of two clinical isolate each for RPV (GenBank accession nos. AB547189 and X98291) and MV (accession nos. AF266288 and JF791787) and 12 PPRV isolates, excluding vaccine strains, (Table 3.1) were used for estimation of evolutionary rate and TMRCA. Furthermore, the coding and noncoding sequences of individual structural genes of PPRV (excluding vaccine strains) were used in this study.

Table 3.1 PPR isolates used for complete genome analysis

Virus isolates	GenBank accession no.	Lineage	Source (reference)
Ivory Coast/1989	EU267273	I	Goat (Chard *et al.*, 2008)
Nigeria/1976	EU267274	II	Sheep (Chard *et al.*, 2008)
Nigeria/1975/1	X74443	II	Goat (Diallo *et al.*, 1994), vaccine strain
Uganda/2012*	KJ867543	III	Goat
UAE/1986*	KJ867545	III	*Dorcas gazelle* (Furley *et al.*, 1987)
Oman/1983*	KJ867544	III	Goat (Taylor *et al.*, 1990)
Ethiopia/1994	KJ867540	III	Goat (Roeder *et al.*, 1994)
Ethiopia/2010*	KJ867541	IV	Goat
India/Sungri/1996*	KJ867542	IV	Goat (provided by Intervet International B.V, Boxmeer, the Netherlands) , vaccine strain
Morocco/2008*	KC594074	IV	Goat (Muniraju *et al.*, 2013)
China/Tibet Bharal/2008	JX217850	IV	Bharal, *Pseudois nayaur* (Bao *et al.*, 2012)
China/Tibet33/2007	JF939201	IV	Goat (Wang *et al.*, 2009)
China/TibetGeg30/2007	FJ905304	IV	Goat (Wang *et al.*, 2009)
Turkey/2000	NC006383	IV	Sheep (Bailey *et al.*, 2005)

*Whole genome sequencing was conducted.

Partial N gene sequences of PPRV isolates (nt positions 1253–1507) that have a detailed history of collection date and place were obtained from GenBank (available up to August 2013). These partial sequences were aligned by using the ClustalW algorithm in BioEdit software v7.2.0. (Hall, 1999) and edited to remove unreliable sequences and/ or regions. Furthermore, identical sequences originating from the same geographic location, host, and year were excluded to avoid redundancy in any subsequent analysis. The final dataset (partial N gene) contained 159 sequences sampled over a period of 45 years (1968–2012).

3.2.4 Selection analysis

The nucleotide and amino acid sequence differences between the PPRV lineages for 12 complete genome sequences were estimated by using BioEdit software v7.2.0. Analyses of selection pressures in individual PPRV genes was performed by obtaining mean ratios of nonsynonymous (dN) to synonymous (dS) substitutions per site. The dN/dS was calculated by using codon-based maximum likelihood approaches with the single-likelihood ancestor method implemented in hypothesis testing using the phylogenies package (Pond *et al.*, 2005) (http://www.datamonkey.org). The proportion of dS substitutions per potential dS site and proportion of dN substitutions per potential dN site were calculated by using the method of Nei and Gojobori (Nei and Gojobori, 1986) and the suite of nucleotide analysis program (www.hiv.lanl.gov).

3.2.5 Bayesian time-scaled phylogenetic analysis

Molecular evolutionary rate and divergence times were co-estimated. A Bayesian maximum clade credibility (MCC) phylogenetic tree was constructed by using Bayesian Markov chain Monte Carlo (MCMC) analysis and Bayesian evolutionary analysis sampling trees (BEAST) software package v1.8.0 (Drummond *et al.*, 2012), and BEAST runs were performed by using the CIPRES Science Gateway (Miller *et al.*, 2010). For each sequence dataset, the best-fit nucleotide substitution model was determined on the basis of Akaike information criterion scores using JModel Test software v2.1.4 (Posada, 2008). An input file for BEAST analysis was obtained by using Bayesian evolutionary analysis utility software v1.8.0, in which sequences were tip dated according to the year of collection. Four molecular clock models

(strict, uncorrelated lognormal distribution [UCLD], uncorrelated exponential distribution [UCED], and random) were tested alongside different demographic models (nonparametric Bayesian skyline plot [BSP] and the parametric constant and exponential growth), and the best models were selected by means of a Bayes factor (BF) test (Kass and Raftery, 1995) using marginal likelihoods values (2lnBF>2) obtained from Tracer v1.5 software (http://beast.bio.ed.ac.uk/tracer).

For each analysis, 2 independent MCMC chains were run to get a final output of 10,000 trees (ESS >200 for all the parameters estimated) and were assessed for their proper mixing, convergence, and consistency by Tracer v1.5 with 10% burn in. The 2 individual runs were combined by using LogCombiner v1.8.0 in the BEAST software package. The nucleotide substitution rate (substitutions/site/year) and the TMRCA (year) values were obtained from Tracer v1.5. The posterior tree distributions were summarized by using TreeAnnotator (http://beast.bio.ed.ac.uk/treeannotator) and exclusion of the first 10% of the trees as burn in. Phylogenetic MCC tree with median node heights were visualized in FigTree software v1.4.0 (http://www.molecularevolution.org/software/phylogenetics/figtree). Furthermore, the demographic history of PPRV was studied by using the partial N gene dataset and less restrictive BSP models in which the changing profile of genetic diversity is plotted against time.

3.2.6 Phylogeographic reconstruction

Bayesian phylogeographic analysis was performed by using complete PPRV genome sequence and partial N gene sequence datasets, and isolates were annotated

according to their location (longitude and latitude). Partial N gene data were chosen instead of F gene data because of increased divergence reported for the N gene (Banyard *et al.*, 2010). For complete genome datasets, sequences from 14 viruses were considered, including 2 vaccine strains (Nigeria 1975/1 and Sungri 1996) to represent all PPRV-endemic areas. Phylogeographic diffusion along the posterior sets of trees and relationships between these locations were identified by using the Bayesian stochastic search variable selection procedure in BEAST v1.8.0 (Lemey *et al.*, 2009). Discrete phylogeographic analysis was performed by using the continuous time Markov chain model and the flexible Bayesian skyride tree.

3.3 Results

3.3.1 Sequence analysis

All 7 PPRV complete genomes (Sungri 1996, Nigeria 75/1, Uganda 2012, Ethiopia 2010, Morocco 2008, UAE 1986 and Oman 1983) were found to be 15,948 nt and so conformed to the rule of 6 as described for all other morbillivirus genomes (Radecke *et al.*, 1995). Resequencing of the Nigeria 75/1 vaccine strain confirmed the sequence for this vaccine and was 100% identical to the previously published sequence (accession no. X7443). The Sungri/96 vaccine strain sequence was also confirmed and completed although 123 nucleotide differences were identified at different locations across the genome when compared to the previously available sequence (accession no. AY560591). The genome organization of all isolates was the same as that for other PPRV strains (as shown in Figure 1.3 and Table 1.2 Chapter 1). The 3' ends of the genomes start with a GP (1 - 107 nt), followed by six transcriptional units of the structural protein genes, N, P, M, F, H and L and end with

the 5' AGP (nucleotide position 15840-15948). The ORF of the P gene also encodes the non-structural proteins C and V. The 5' UTR of M gene and 3'UTR of F gene together was 1080 nt long and extremely GC rich (66-68%). The untranslated regions typically contain the gene end signal of the previous gene followed by an IG tri-nucleotide sequence (CTT) and the gene start signal of the subsequent gene. The sequences flanking the IG tri-nucleotide (10 nt) are conserved between the N/ P and P/ M junctions among all the PPRV isolates.

Phylogenetic analysis of the complete genome sequences of PPRV clustered the sequences into 4 lineages. The complete genomes of PPRV isolates from Ethiopia 1994, Oman 1983, UAE 1986, and Uganda 2012 sequenced in this study belonged to lineage III and the isolates Sungri 1996, Morocco 2008, and Ethiopia 2010 belong to lineage IV. Comparison of the 12 (excluding Nigeria 1975/1 and Sungri 1996 vaccine strains) aligned complete genome sequences showed that nucleotide differences ranged from 0.1% to 11.9%, and amino acid differences ranged from 0.1% to 7.2% (Table 3.2).

Further, the selection analysis was performed on PPRV genes. The dN/dS for coding regions of the various genes of PPRV (n = 12) for all 4 lineages ranged from 0.06 to 0.45 (Table 3.3). The dN/dS per site across the coding region of different genes of PPRV genome obtained by Nei and Gojobori method are shown in Figure 3.1. The highest dN/dS ratio was observed in the P gene, followed by the H, N, F, L, and M genes. The relative nucleotide substitution rates at all 3 codon positions of the structural genes of PPRV showed that substitutions were more frequent at the third codon position (Table 3.3) as expected.

Table 3.2 Nucleotide and amino acid sequence differences in complete genomes of PPRV lineages

PPRV lineages	I	II	III	IV
I		5.1	6.1-7.0	5.7-6.1
II	**9.0**		5.7-6.3	4.0-4.2
III	**10.9-11-9**	**9.9-10.8**	0.2-3.0 **0.2-6.2**	6.1-7.2
IV	**10.3-10.7**	**7.2-7.6**	**10.7-11.8**	0.1-2.0 **0.1-3.2**

*Values are percentage nucleotide (bold) and amino acid sequences differences.

Table 3.3 Nucleotide substitution rates at codon positions of PPRV genes by BEAST analysis and dN/dS by single-likelihood ancestor counting (SLAC)

Gene	Total amino acids	Codon position			Mean dN/dS
		CP1.mu	CP2.mu	CP3.mu	
N	526	0.44	0.33	2.23	0.13
P	510	0.81	0.69	1.49	0.45
M	336	0.48	0.15	2.36	0.06
F	547	0.46	0.26	2.29	0.10
H	610	0.57	0.37	2.06	0.19
L	2184	0.42	0.18	2.40	0.08

*BEAST, Bayesian evolutionary analysis sampling trees; dN/dS, nonsynonymous/ synonymous substitutions per site; CP, codon position.

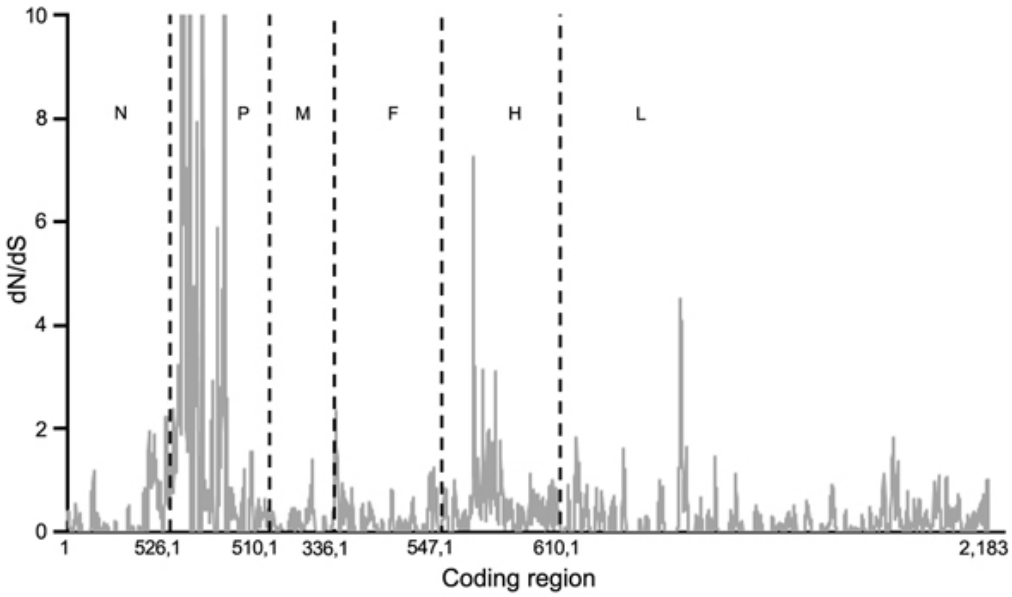

Figure 3.1 Mean ratios of nonsynonymous (dN) to synonymous (dS) substitutions per site of concatenated coding regions of PPRV genome. Vertical dashed lines indicate gene junctions with sliding windows of size = 5 codons. dN/dS values ≥ 10 are shown as 10. Numbers along baseline indicate coding regions (base pairs) of individual genes.

3.3.2 Evolutionary rate estimates

Complete genome sequences of 12 PPRV and partial N gene dataset (n = 159) were analyzed by using the coalescent-based Bayesian MCMC approach. The general time-reversible (GTR) nucleotide substitution model with a gamma distribution for rate variation was selected on the basis of Akaike information criterion scores. Bayes factor tests with marginal likelihood comparisons showed that the relaxed UCED clock model best fitted the PPRV complete genome and partial N gene datasets (Table 3.4). The 2lnBF value was >78 between UCED and strict clocks and 2–6 between UCED/ UCLD and UCED/ random clocks, which provided strong evidence for the UCED clock model. There was no difference between different demographic models compared within the UCED clock model (2lnBF <2). However, the exponential demographic model was chosen because it provided a narrow margin of 95% highest posterior density (HPD) estimates.

Table 3.4 Bayesian Markov chain Monte Carlo analysis for genomes of PPRV.

Sequence dataset (no.)	Models, Substitution/ clock/ demographic	Mean nucleotide substitution rate, substitutions/site/y (95% HPD)	TMRCA, y (95% HPD)	Bayes factor, -log likelihood	2lnBF
PPRV Complete genome (12)	GTR+G/ strict/ BSP	3.2×10^{-4} (2.02×10^{-4} - 4.31×10^{-4})	1763 (1653-1832)	-46972.98	-93945.96
	GTR+G/ strict/ CS	3.21×10^{-4} (2.12×10^{-4} - 4.38×10^{-4})	1763 (1659-1834)	-46973.06	-93946.12
	GTR+G/ strict/ EG	3.24×10^{-4} (2.12×10^{-4} - 4.33×10^{-4})	1765 (1668-1836)	-46973.06	-93946.12
	GTR+G/ UCLD/ BSP	2.89×10^{-3} (3.21×10^{-8} - 6.92×10^{-4})	1691 (123_{BCE}-1944_{CE})	-46935.66	-93871.32
	GTR+G/ UCLD/ CS	3.03×10^{-4} (8.99×10^{-9} - 7.07×10^{-4})	1705 (123-1961)	-46935.86	-93871.72
	GTR+G/ UCLD/ EG	3.72×10^{-4} (3.01×10^{-5} - 7.93×10^{-4})	1767 (1222-1948)	-46935.89	-93871.78
	GTR+G/ UCED/ BSP	7.91×10^{-4} (7.46×10^{-5} - 1.53×10^{-3})	1889 (1586-1968)	-46933.82	-93867.64
	GTR+G/ UCED/ CS	7.98×10^{-4} (8.03×10^{-5} - 1.54×10^{-3})	1887 (1569-1968)	-46933.98	-93867.96
	GTR+G/ UCED/ EG	**9.09×10^{-4} (2.13×10^{-4} - 1.64×10^{-3})**	**1904 (1730-1966)**	**-46933.96**	**-93867.92**
	GTR+G/ random/ BSP	7.01×10^{-4} (5.55×10^{-4} - 8.50×10^{-4})	1888 (1862-1908)	-46934.75	-93869.5
	GTR+G/ random/ CS	6.97×10^{-4} (5.38×10^{-4} - 8.41×10^{-4})	1887 (1860-1908)	-46934.64	-93869.28
	GTR+G/ random/ EG	7.04×10^{-4} (5.57×10^{-4} - 8.57×10^{-4})	1888 (1861-1908)	-46934.89	-93869.78
N partial (159)	GTR+G/ strict/ BSP	1.22×10^{-3} (9.39×10^{-4} - 1.51×10^{-3})	1890 (1857-1917)	-2884.524	-5769.048
	GTR+G/ strict/ CS	1.23×10^{-3} (9.49×10^{-4} - 1.52×10^{-3})	1886 (1853-1913)	-2887.723	-5775.446
	GTR+G/ strict/ EG	1.24×10^{-3} (9.71×10^{-4} - 1.56×10^{-3})	1893 (1863-1919)	-2885.44	-5770.88
	GTR+G/ UCLD/ BSP	1.45×10^{-3} (1.06×10^{-3} - 1.87×10^{-3})	1896 (1815-1943)	-2806.535	-5613.07
	GTR+G/ UCLD/ CS	1.41×10^{-3} (1.05×10^{-3} - 1.80×10^{-3})	1882 (1793-1935)	-2805.535	-5611.07
	GTR+G/ UCLD/ EG	1.49×10^{-3} (1.10×10^{-3} - 1.89×10^{-3})	1904 (1838-1943)	-2805.921	-5611.842
	GTR+G/ UCED/ BSP	1.52×10^{-3} (1.11×10^{-3} - 1.98×10^{-3})	1904 (1817-1949)	-2799.572	-5599.144
	GTR+G/ UCED/ CS	1.46×10^{-3} (1.05×10^{-3} - 1.88×10^{-3})	1886 (1785-1940)	-2799.512	-5599.024
	GTR+G/ UCED/ EG	**1.56×10^{-3} (1.16×10^{-3} - 1.99×10^{-3})**	**1910 (1846-1947)**	**-2799.444**	**-5598.888**
	GTR+G/ random/ BSP	1.26×10^{-3} (9.44×10^{-4} - 1.58×10^{-3})	1881 (1837-1915)	-2865.846	-5731.692
	GTR+G/ random/ CS	1.24×10^{-3} (9.38×10^{-4} - 1.57×10^{-3})	1875 (1831-1910)	-2866.111	-5732.222
	GTR+G/ random/ EG	1.27×10^{-3} (9.62×10^{-4} - 1.60×10^{-3})	1880 (1841-1914)	-2866.929	-5733.858
NCDS (12)	GTR+G/ UCED/ EG	1.01×10^{-3} (2.79×10^{-4} - 1.83×10^{-3})	1924 (1799-1970)	NA	NA
N gene (12)	GTR+G/ UCED/ EG	1.08×10^{-3} (3.19×10^{-4} - 1.93×10^{-3})	1923 (1804-1970)	NA	NA
P CDS (12)	GTR+I/ UCED/ EG	1.11×10^{-3} (3.46×10^{-4} - 1.29×10^{-3})	1931 (1833-1972)	NA	NA
P gene (12)	GTR+I/ UCED/ EG	1.19×10^{-3} (3.46×10^{-4} - 2.03×10^{-3})	1930 (1828-1971)	NA	NA
M CDS (12)	GTR+G/ UCED/ EG	6.52×10^{-4} (1.20×10^{-4} - 1.20×10^{-3})	1897 (1695-1964)	NA	NA
M gene (12)	GTR+I/ UCED/ EG	2.49×10^{-3} (9.96×10^{-4} - 4.14×10^{-3})	1944 (1879-1973)	NA	NA
FCDS (12)	GTR+I/ UCED/ EG	8.95×10^{-4} (2.43×10^{-4} - 1.58×10^{-3})	1914 (1766-1968)	NA	NA
F gene (12)	GTR+G/ UCED/ EG	1.33×10^{-3} (3.26×10^{-4} - 2.36×10^{-3})	1912 (1754-1967)	NA	NA
H CDS (12)	GTR+G/ UCED/ EG	1.21×10^{-3} (3.96×10^{-4} - 2.04×10^{-3})	1926 (1826-1969)	NA	NA
H gene (12)	GTR+G/ UCED/ EG	1.25×10^{-3} (4.34×10^{-4} - 2.14×10^{-3})	1925 (1821-1968)	NA	NA
L CDS (12)	GTR+I/ UCED/ EG	9.82×10^{-4} (3.76×10^{-4} - 1.67×10^{-3})	1929 (1834-1969)	NA	NA
L gene (12)	GTR+I/ UCED/ EG	9.69×10^{-4} (3.36×10^{-4} - 1.64×10^{-3})	1927 (1820-1969)	NA	NA
PPRV/RPV/ MV (16)	GTR+G+I/ UCED/ EG	1.89×10^{-3} (5.55×10^{-4} - 3.31×10^{-3})	1616 (1072-1859)	NA	NA

Bold indicates best-fit models. HPD, highest posterior density; TMRCA, time to most recent common ancestor; GTR+G, general time-reversible with gamma distribution rates; BSP, Bayesian skyline plot; CS, constant size; EG, exponential growth; UCLD, uncorrelated lognormal distribution; UCED, uncorrelated exponential distribution; NA, not applicable; GTR + I, general time-reversible with invariant sites.

Accordingly, the UCED and exponential growth model have been directly used for the individual PPRV gene dataset and the PPRV/RPV/MV complete genome dataset to estimate the TMRCA and substitution rate per site per year. When we used the UCED and exponential growth models, we found that the mean evolutionary substitution rate of the PPRV complete genome was estimated to be 9.09×10^{-4} (95% HPD 2.13×10^{-4}–1.64×10^{-3}). When 2 complete genome sequences of vaccine strains were added into this analysis, the same models (GTR nucleotide substitution model with a gamma distribution, UCED, and the exponential growth demographic models) were best fitted, and the mean substitution rate/site/year was reduced to 7.86×10^{-4} (95% HPD 2.17×10^{-4}–1.4×10^{-3}). Furthermore, the evolutionary nucleotide substitution rate for combined PPRV/RPV/MV complete genomes was 1.89×10^{-3} (95% HPD 5.55×10^{-4}–3.31×10^{-3}). Analysis of individual genes of the PPRV coding region dataset, coding and noncoding region datasets, and partial N gene dataset are shown in Table 3. 4.

3.3.3 Temporal dynamics

A Bayesian time-scaled MCC tree based on complete PPRV genomes was constructed (Figure 3.2) by using the UCED model with exponential growth demography. The estimated median TMRCA of PPRV for all 4 lineages and divergence of lineage III PPRV were found to be approximately 1904 (95% HPD 1730–1966). Lineage I diverged in around 1939 (95% HPD 1843–1970). Lineages II and IV diverged from each other in around 1956 (95% HPD 1885–1973). The TMRCA for lineage III viruses (n = 4) used in this study was estimated to be around1956 (95% HPD 1887–1978). TMRCA for lineages I and II PPRV were not predicted because only 1 virus from each lineage was used. The TMRCA for lineage

IV viruses (n = 6) used in this study was estimated to be around 1987 (95% HPD 1957–1998). When both Nigeria 1975/1 and Sungri 1996 vaccine strains were included in the study, the TMRCA for all lineages of PPRV shifted from 1904 (95% HPD 1730–1966) to 1891 (95% HPD 1705–1960). Analysis of the partial N gene dataset showed the TMRCA as 1910 (95% HPD 1846–1947) for all lineages of PPRV, 1960 (95% HPD 1941–1971) for lineage III, 1958 (95% HPD 1946–1971) for lineage I, 1961 (95% HPD 1941–1967) for lineage II, and 1987 (95% HPD 1969–1988) for lineage IV.

Results of TMRCA analysis using complete coding and coding and noncoding regions of individual PPRV genes are shown in Table 3.4. If one compares the difference in TMRCA between coding and noncoding sequences of individual genes in the analysis, the large change in TMRCA was found only for the M gene (i.e., 1944, 95% HPD 1879–1973). The TMRCA of PPRV/RPV/MV was estimated to be around 1616 (95% HPD 1072–1859), and the TMRCA for PPRV was estimated to be 1931 (95% HPD 1858–1956) (Figure 3.3).

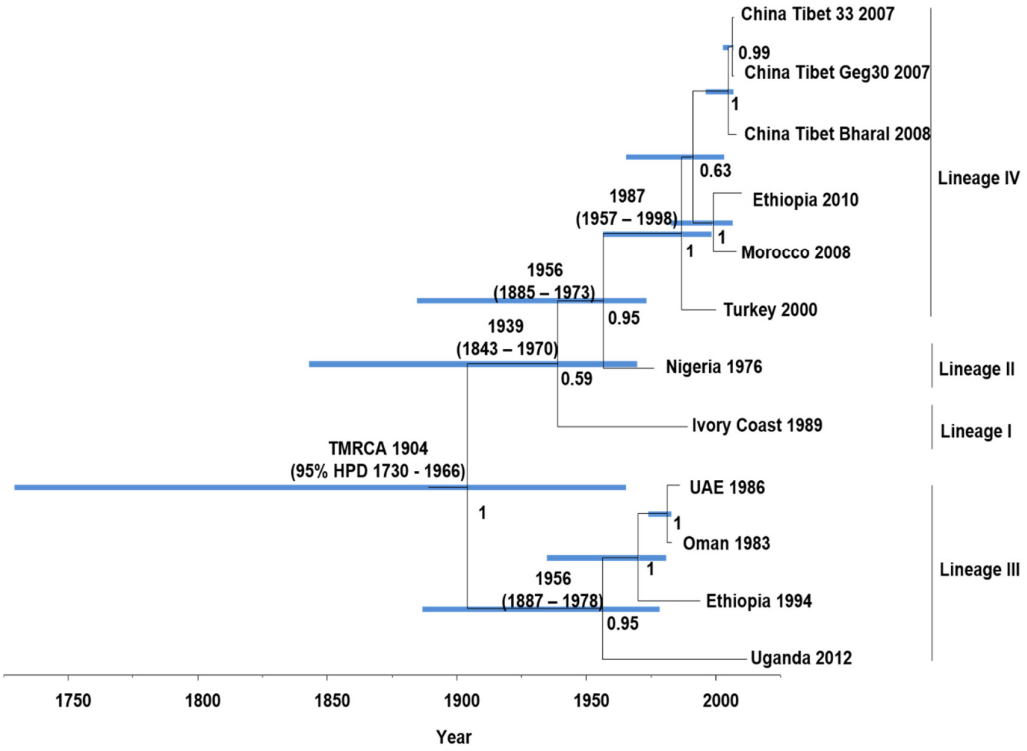

Figure 3.2 Time-scaled Bayesian maximum clade credibility phylogeny tree based on PPRV complete genome sequences. The tree was constructed by using the uncorrelated exponential distribution model and exponential tree prior. Branch tips correspond to the date of collection and branch lengths reflect elapsed time. Tree nodes were annotated with posterior probability values and estimated median dates of time to most recent common ancestor (TMRCA). Corresponding 95% highest posterior density (HPD) interval values of TMRCA are indicated as blue bars. Horizontal axis indicates time in years. UAE, United Arab Emirates.

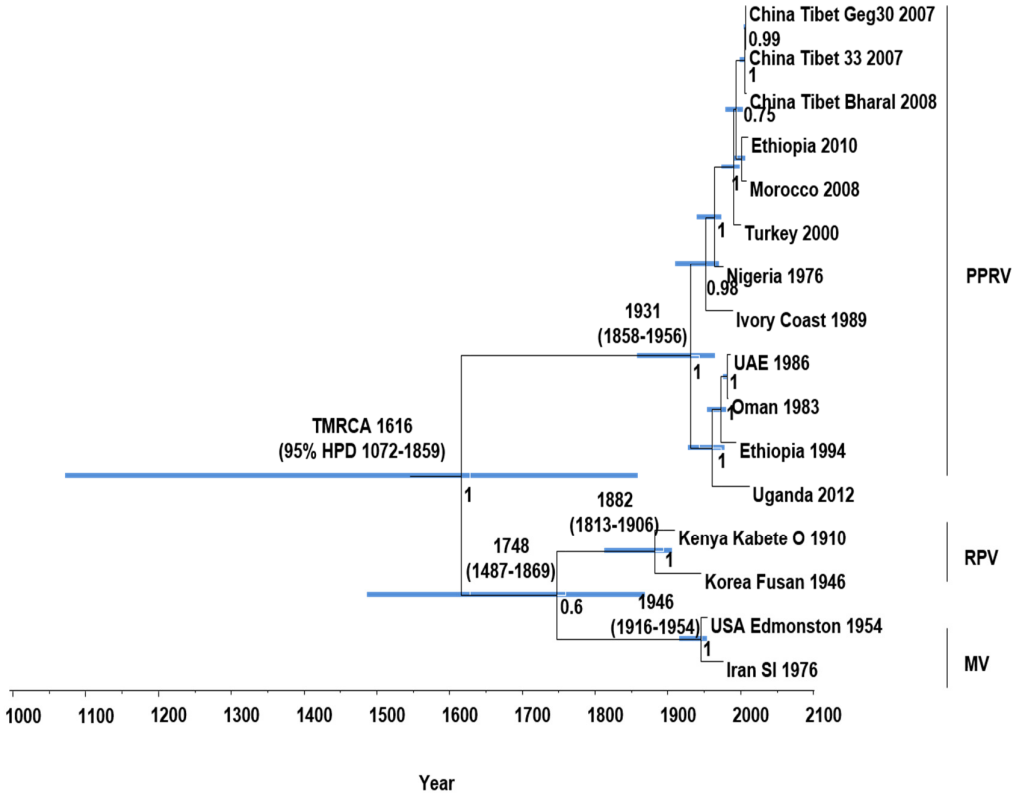

Figure 3.3 Time-scaled Bayesian maximum clade credibility phylogeny tree based on PPRV, RPV and MV complete genome sequences. The tree was constructed by using the uncorrelated exponential distribution model and exponential tree prior. Branch tips correspond to the date of collection and branch lengths reflect elapsed time. Tree nodes were annotated with posterior probability values, estimated median dates of time to most recent common ancestor (TMRCA). Corresponding 95% highest posterior density (HPD) values of TMRCA are indicated as blue bars. Horizontal axis indicates time in years. UAE, United Arab Emirates.

3.3.4 Population demography of PPRV

The demographic history of PPRV was investigated by using the partial N gene sequence dataset according to the BSP method implemented in BEAST. The BSP with an assumed piecewise-constant model has facilitated estimation of effective population size through time. The BSP showed that the population did not show much genetic diversity (effective number of infections) until the mid-1990s when the diversity started to increase. Toward the first decade of the 21st century, the population size appeared to reach a peak and then showed a small decrease until the most recent sampling in 2012 (Figure 3.4). The HPD interval size for the plot is narrow (closer to median genetic diversity), which indicates strong support for this population trend.

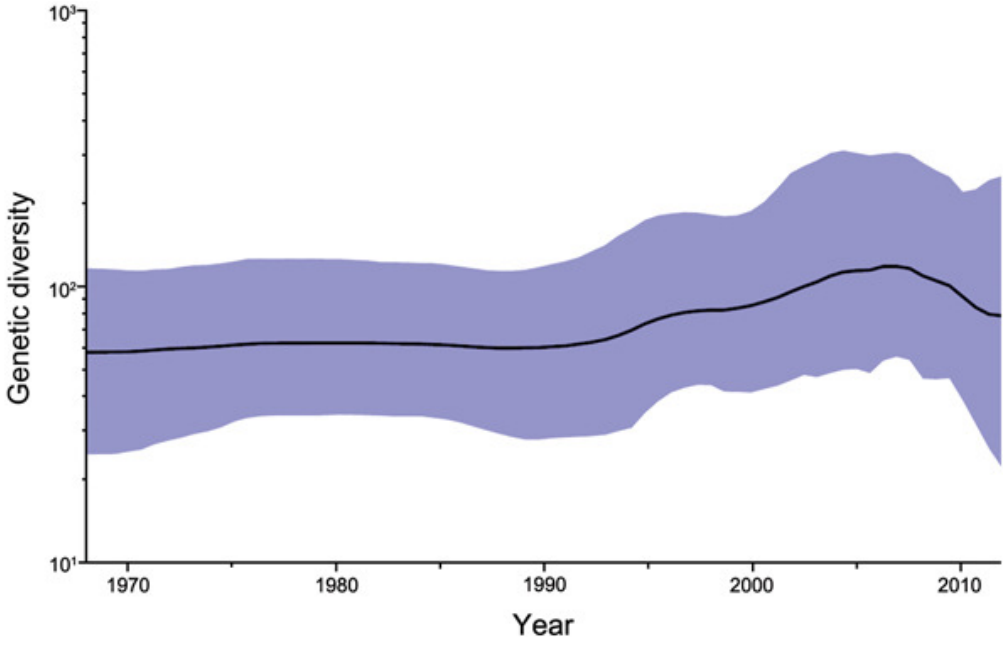

Figure 3.4 Bayesian skyline plot showing demographic history of global PPRVs sampled during 1968–2012. Genetic diversity was estimated by using a partial nucleoprotein gene dataset (n = 159). The thick black line represents median genetic diversity and the blue shaded areas show 95% highest posterior density estimate.

3.3.5 Phylogeographic analysis

To estimate the geographic origin of PPRV, the results of Bayesian phylogeographic analyses was summarised and visualised using annotated MCC tree (Figure 3.5). The complete genome sequence data used in this analysis incorporated all 14 isolates, including the vaccine strains, from 10 discrete locations so as not to leave out any reported virus-endemic area. The root state posterior probabilities for all the locations ranged between 9.02% and 12.69%; Nigeria and the Ivory Coast receiving marginally higher support, 12.69% and 10.53%, respectively, than the rest of the locations (Figure 3.5).

Because the geographic origin of PPRV could not be localized to a single country by using 14 complete genome sequences, further phylogeographic analysis was performed by using 159 partial N gene sequences collected from 30 locations during 1968–2012. The root state posterior probabilities of PPRV ranged from 0.11% to 17.20%, and Nigeria (17.20%), Ghana (14.28%), and Sierra Leone (11.68%) showed the highest marginal support (Figure 3.6). The highest marginal support of root state posterior probabilities indicated that the geographic origin of lineage I PPRV was Senegal (27.44%), that of lineage II PPRV was Nigeria (27.00%), that of lineage III PPRV was Sudan (30.73%), and that of lineage IV PPRV was India (36.00%).

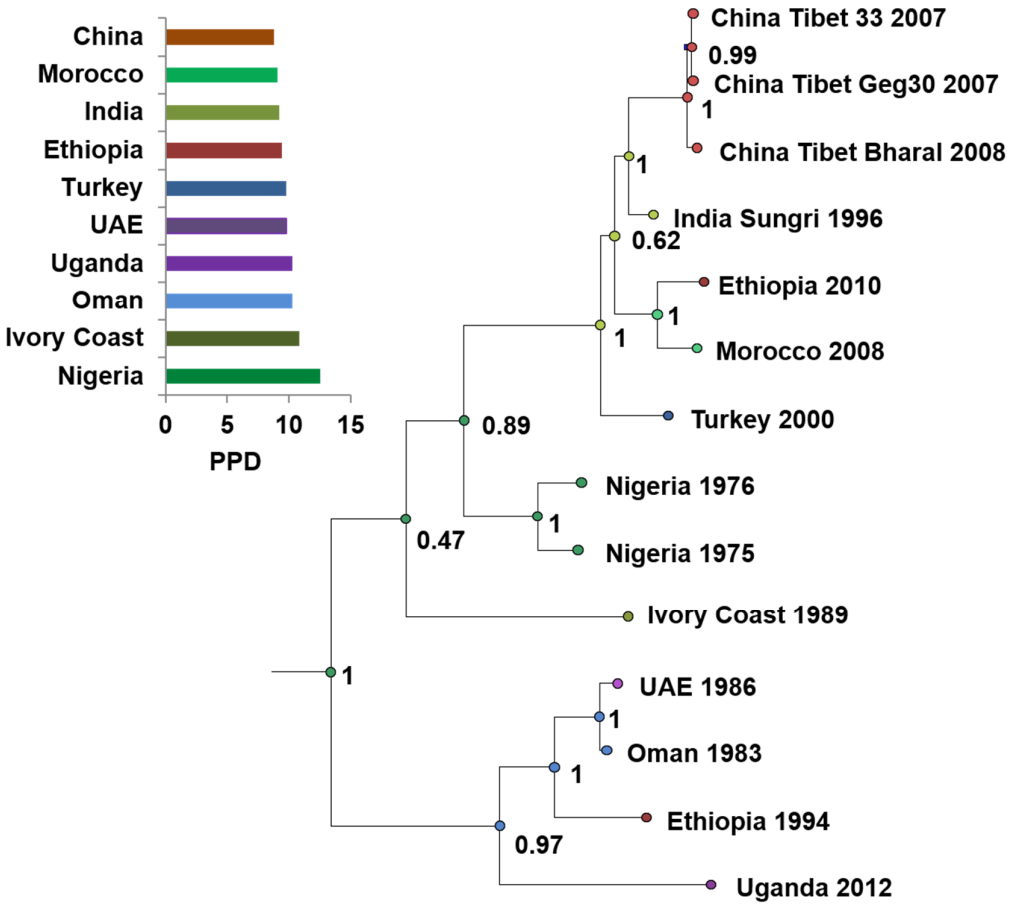

Figure 3.5 Maximum clade credibility tree constructed for the geospatial analysis of PPRVs by using complete genome data. Nodes are coloured according to the most probable location of their ascendant locations. Posterior probability values are shown along tree nodes. Posterior probability distribution (PPD) values of root location states of the ancestral node are shown along the x-axis at the top left. UAE, United Arab Emirates.

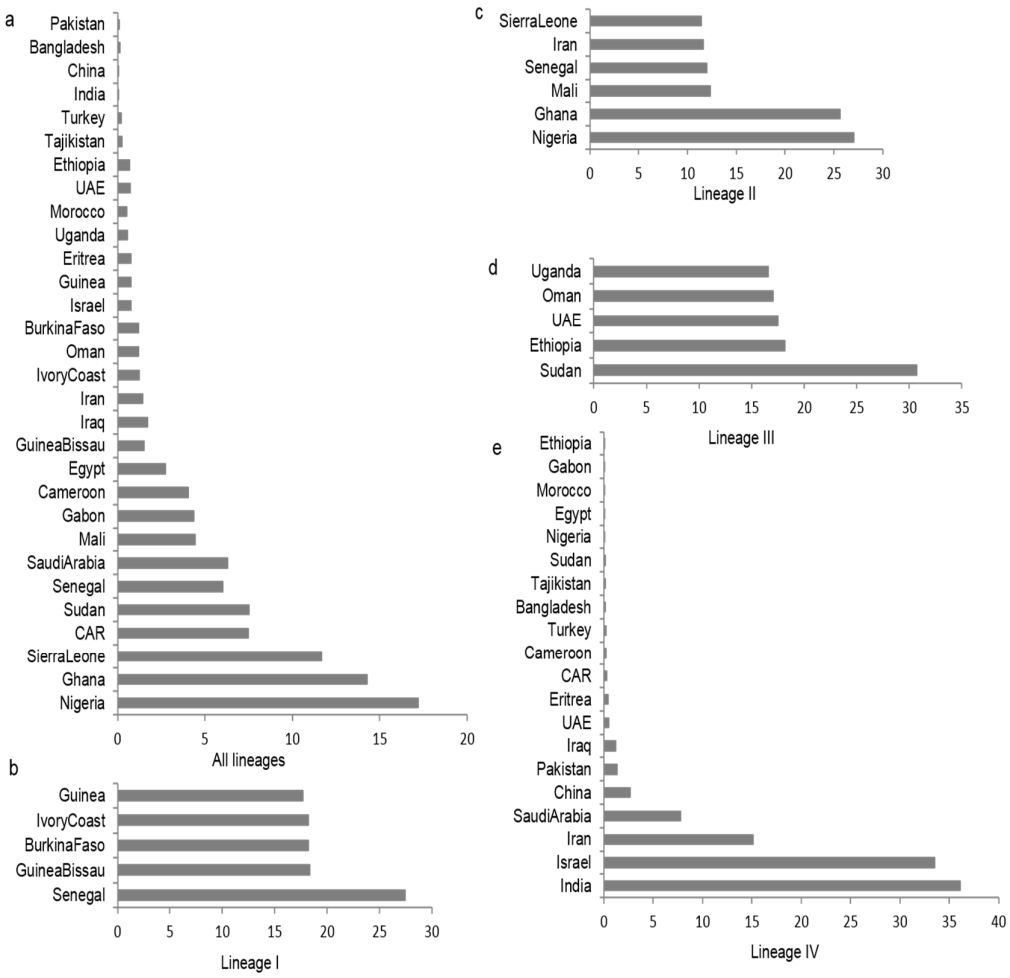

Figure 3.6 Probability of root locations of the most recent common ancestral PPRV. MCC trees were obtained by using the continuous time Markov chain and Bayesian stochastic search variable selection procedures. Root location probabilities of the most recent common ancestor using global PPRV isolates (panel A) are shown graphically alongside lineages I–IV (panels B–E) and were estimated by using a complete dataset of PPRV partial nucleoprotein gene data and individual lineages separately. Probabilities of root locations are shown as percentages along the x-axes.

3.4 Discussion

The complete genomes of 3 lineage III (Uganda 2012, UAE 1986 and Oman 1983) and 3 lineage IV (Ethiopia 2010, Morocco 2008, and Sungri 1996) isolates and 1 lineage II (Nigeria 1975/1) of PPRV were sequenced in this study. Partial genome sequence of PPRV Sungri 96 isolate was available at the time of study in GenBank (accession no AY560591), that lacked genome promoter sequence and with interspersed nucleotide deletions in M/F gene junction. PPRV genome termini determined by tailing of cDNA with cytosine residues using terminal deoxy-nucleotidyl transferase (Bailey *et al.*, 2005; Chard *et al.*, 2008) was not found efficient to generate the sequences of 3' and 5' termini. An improved method was adopted here to determine PPRV genome 3' and 5' termini as described for closely related paramyxovirus genome (Li *et al.*, 2005; Tillett *et al.*, 2000) and this had been proved to be efficient as the seven PPRV genome termini were successfully determined. Recently, this novel method was also adapted by other researchers to determine PPRV genome termini (Dundon *et al.*, 2014). Another difficult region to sequence in PPRV genome is the M/F gene junction, which is rich in GC content and not easily amplifiable and as such clean and acceptable chromatograph peaks are difficult to achieve using Sanger sequencing. During this study, it was found that the designing of primer sets at appropriate locations is the critical factor that determines the amplification followed by sequencing.

Newly sequenced genomes and other available genomes in GenBank were utilised to assess the evolutionary substitution rate, TMRCA, and divergence of PPRV lineages and the geographic origin of PPRV. To the best of my knowledge, this is the first

study on the molecular evolutionary dynamics of PPRV using full genome sequences from all four lineages of PPRV.

The measure of selective pressures acting across the PPRV genome showed only purifying (stabilizing) selection occurring across the genome and no evidence of positive selection. The conservation of amino acid residues was further confirmed by the fact that the relative substitution rates at the third codon position of all the genes were higher than those for the first and second codon positions. The observed upper limit of 11.9% nt divergence (7.2% aa divergence) among PPRVs is consistent with the low level of antigenic divergence observed because despite lineage differentiation, only a single serotype exists for PPRV. Homologous recombination events are generally rare or absent in negative-sense RNA viruses (Han and Worobey, 2011) and thus could not have been evaluated in this study.

From a genetic perspective, substitution rates are critical parameters for understanding virus evolution, given that restrictions in genetic variation within a population of viruses can lead to lower adaptability and pathogenicity (Denison *et al.*, 2011). Our analyses estimated a range of PPRV nucleotide substitution rates throughout the complete genome of 1.64×10^{-3}–2.13×10^{-4} substitutions/site/year, which is similar to that predicted for other paramyxoviruses (10^{-3}–10^{-4} substitutions/site/year) (Furuse *et al.*, 2010; Jenkins *et al.*, 2002; Pomeroy *et al.*, 2008; Wertheim and Pond, 2011). Despite low levels of antigenic divergence, as shown by the existence of a single serotype, the genome plasticity of PPRV might explain its ability to emerge and adapt in new geographic regions and hosts, as reported extensively across vast areas in recent years. The TMRCA of PPRV

obtained from complete genome sequence was estimated to be during 1904 (95% HPD 1730–1966). Similarly, the estimated TMRCA obtained from individual gene sequence, partial N gene sequence of PPRV, and combined PPRV/RPV/MV complete genome sequences was during 1910–1944.

That the predicted TMRCA for PPRV was during the early 20th century is reasonable because the first recorded description of PPRV was made in 1942 (Gargadennec and Lalanne, 1942). The delay of a few decades before identification of PPRV as a distinct viral entity after its initial detection can likely be attributed to confusion in differentiation between PPRV and RPV, a virus for which extensive cross-neutralization is observed after vaccination and natural infection, and lack of differentiating diagnostic tools. Substitution rates were consistent across each gene for PPRV. However, greater substitution rates were observed in the GC rich regions of the F and M genes. Similarly, the substitution rate was greater, as predicted because of the variability seen at the nucleotide level, in the highly variable region of the N gene sequence (255 nt) for PPRV. The TMRCA estimation was not possible for lineage I and II viruses (the lineage II Nigeria 75/1 vaccine strain was omitted due to its passage attenuation) because only 1 complete genome sequence was available for each lineage. Therefore, more complete genome sequences are required to study evolutionary and phylogenetic relationships for these lineages.

Biased estimates in substitution rate and TMRCA were observed by using datasets that included tissue culture–passaged, attenuated vaccine strain complete genome sequences, in which slower evolutionary substitution rates and earlier TMRCA were predicted. Similar observations were reported for PPRV/RPV/MV N gene sequence

analyses, in which a slower and biased nucleotide substitution rate was observed when vaccine strain sequences (Furuse *et al.*, 2010) were included in the analysis and faster substitution rates and later TMRCA predictions were suggested when vaccine strain sequence data were excluded (Wertheim and Pond, 2011).

Spatial and temporal dynamics of RNA viruses are often reflected by their phylogenetic structure (Biek *et al.*, 2006). Potential divergence events for different PPRV lineages were inferred by using rooted, time-measured phylogenetic trees with higher confidence from the PPRV complete genome sequence dataset. The inferred phylogeny supports the initial divergence of lineage III isolates, followed by lineage I isolates; lineage II and IV isolates were predicted to have diverged from each other at a later time. The inference of divergence events presented facilitated a better understanding of historical divergence of PPRV and offered further opportunities to study viral demographic history and dispersal events.

The demographic analysis of PPRV with the BSP indicated historically constant genetic variability of PPRV over time. This finding could be a reflection of the use of RPV vaccine in small ruminants to protect animals against PPRV through the 1990s, which might have affected the evolution and spread of PPRV. In the early 21st century, genetic diversity of PPRV has gradually increased, which reflects frequent outbreak reports. The increased genetic diversity may be a driver for selection pressures within individual lineages and might result in extinction events, as suggested by an absence of lineage I virus. In recent years, as efforts have

increased to actively control and eradicate PPRV, a decrease in genetic diversity has been observed.

Phylogeographic reconstruction with spatial and temporal information of virus isolates has enabled an understanding of the historic emergence and dispersal patterns involved in virus evolution (Lemey *et al.*, 2009). Although PPRV existed earlier than its first description in Ivory Coast in 1942 (Diallo, 1988), PPRV was later reported in Senegal, Chad, Togo, Benin, Ghana, Nigeria, Oman, Sudan, Saudi Arabia, India, Jordan, Israel, Ethiopia, Kenya, Uganda, and Pakistan (Sen *et al.*, 2010). Our phylogeographic analysis indicated that Nigeria was the geographic origin of the most recent common ancestor of PPRV because of the highest root location state probability. Furthermore, geographic origins of the most recent common ancestor of PPRV lineages I, II, and III were predicted to be across Africa; lineage IV likely emerged in India. In conclusion, these findings suggest that the origin of PPRV was in western Africa, which then spread to eastern Africa, the Middle East, and Asia. However, although these predictions are suggestive of a potential origin for PPRV, caution must be exercised in their interpretation because estimates of geographic origin rely on available datasets, and these datasets need enhancing to provide greater confidence for phylogenetic assessment. As more sequence data become available for PPRV and the other morbilliviruses, ancestral origins of each virus and intraspecies differentiation might become clearer.

Chapter 4 Establishment of reverse genetics system to rescue the Nigeria 75/1 PPR vaccine strain for the development of marker vaccines

4.1 Introduction

Across the developing world, PPRV places a huge disease burden on agriculture, primarily affecting the production and sustainability of small ruminant farming. The disease is most effectively controlled by vaccinating sheep and goats with live attenuated vaccines that provide lifelong immunity. However, the current vaccines and serological tests are unable to enable DIVA. This factor precludes the meaningful assessment of vaccine coverage and epidemiological surveillance based on serology, in turn reducing the efficiency of control programs. The availability of a recombinant PPRV vaccine with a proven functionality is a prerequisite for the development of novel vaccines that may enable the development of DIVA tools for PPRV diagnostics. This Chapter describes the establishment of reverse genetics technique for PPRV that provides a means to manipulate RNA virus genomes through DNA copies (cDNA) of the RNA genome. Further, the rescue of the positively marked recombinant virus by insertion of eGFP and the negatively marked recombinant virus by mutation of C77 mAb binding epitope on H gene of PPRV.

4.2 Materials and methods

4.2.1 Design and synthesis of PPRV Nigeria 75/1 full-genome cDNA incorporating eGFP

The full-length PPRV cDNA plasmids generated were based on the PPRV Nigeria 75/1 vaccine strain (GenBank accession no X74443). The plasmid containing the

complete PPRV antigenome (positive sense strand) sequence (15948 nt) with the insertion of eGFP gene was designed and synthesised commercially (DNA2.0, USA) (Figure 4.1a and Appendix VII). The eGFP reporter gene (822 nt) was introduced to enable rapid evaluation of rescue events, as a separate transcriptional unit between the P and M gene with the authentic 5' UTR of the M gene and the 3' UTR of the P gene. Unique restriction enzyme sites were inserted by nucleotide substitution into the UTRs of each gene and as such did not affect the total genome length or viral protein sequences. The full length clone was under the expression control of the T7 RNA polymerase promoter and the primary transcript was cleaved at the AGP by the hepatitis delta ribozyme (Figure 4.1a). The synthesised plasmid, pPPRV+GFP Nigeria 75/1 was sequenced in its entirety to ensure the sequence was 100% identical to the wild type vaccine strain.

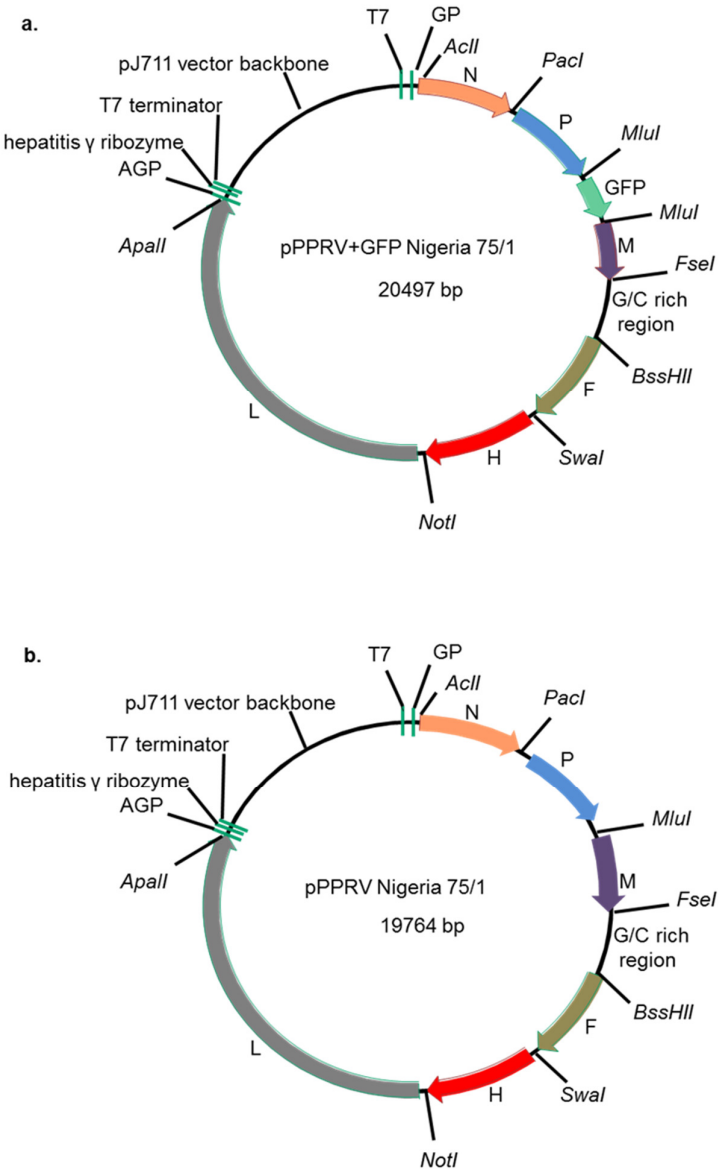

Figure 4.1 Schematic representation of the PPRV recombinant plasmids. (a) the synthetic plasmid, pPPRV+GFP Nigeria 75/1, incorporating eGFP as a reporter gene between the P and M genes and restriction enzyme sequences. (b) plasmid pPPRV Nigeria 75/1 without the eGFP gene incorporating restriction enzyme sequences. GP represents genome promoter and AGP represents anti-genome promoter.

4.2.2 Construction of full-length PPRV Nigeria 75/1 cDNA plasmid with mutations at C77 monoclonal antibody binding site

Initially, the eGFP gene in the plasmid pPPRV+GFP Nigeria 75/1 was removed using *Mlu*I to obtain pPPRV Nigeria 75/1 (Figure 4.1b). The C77 mAb binding site mapped previously through phage display peptide library screening (Buczkowski, 2010) was altered in the plasmid pPPRV Nigeria 75/1 by SDM to obtain three mutated plasmids (pPPRV-C77a, pPPRV-C77b and pPPRV-C77c). The C77 epitope mapped on the H protein of PPRV by phage display peptide library screening is depicted in Figure 4.2. Further, the 3-dimensional (3D) structure of H protein of PPRV Nigeria 75/1 highlighting critical residues in the C77 mAb binding epitope are shown in Figure 4.3. The amino acid residues critical for C77 mAb binding were mutated (6 amino acids in the pPPRV-C77a Nigeria 75/1, 3 amino acids in the pPPRV-C77b Nigeria 75/1 and 3 amino acids in the pPPRV-C77c Nigeria 75/1) into alanine (A) residues on the H gene of the pPPRV Nigeria 75/1 full-length cDNA plasmid through SDM using overlapping primer sets (Appendix III). An intermediate cloning vector pT7 Blue (Promega) containing the H gene from pPPRV Nigeria 75/1 was constructed and used to substitute either three or six amino acids as shown in the Figure 4.2. The H gene of the PPRV full-length plasmid was replaced with the mutated H genes using *Not*I and *Swa*I restriction enzyme sites. This resulted in the generation of 3 different full-length plasmids of PPRV Nigeria 75/1:

1. pPPRV-C77a Nigeria 75/1 Y540A I542A Y543A R547A S549A S550A
2. pPPRV-C77b Nigeria 75/1 Y540A I542A Y543A
3. pPPRV-C77c Nigeria 75/1 R547A S549A S550A

```
                                                          535     * **   * **     555
PPRV Nig75/1 H                                              EHAIVYYIYDTGRSSSYFYPV
pPPRV-C77a Nig75/1 Y540A I542A Y543A R547A S549A S550A      EHAIVAYAADTGASAAYFYPV
pPPRV-C77b Nig75/1 Y540A I542A Y543A                        EHAIVAYAADTGRSSSYFYPV
pPPRV-C77c Nig75/1 R547A S549A S550A                        EHAIVYYIYDTGASAAYFYPV
```

Figure 4.2 Schematic representation of predicted epitope for C77 mAb binding. The proposed epitope (upper strand) for the C77 mAb binding site on H protein of PPRV Nigeria 75/1 as determined by phage display, and the mutated H (lower strands). Residues critical for C77 mAb binding are indicated by the star symbols and the amino acids position on H protein are indicated by number. Amino acid residues mutated to alanine (A) in full-length plasmids are coloured red and underlined in lower strands.

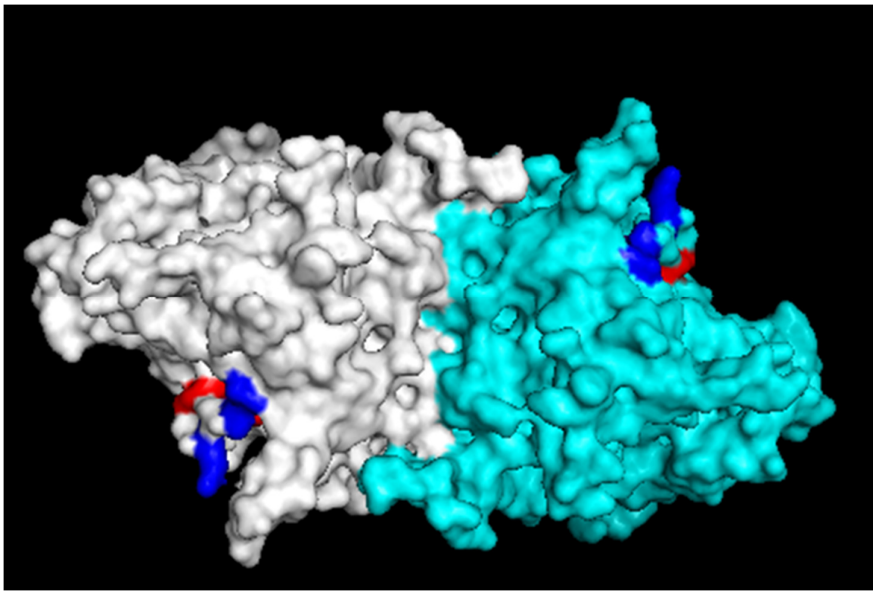

Figure 4.3 The 3D structure of H protein of PPRV Nigeria 75/1 highlighting the C77 mAb binding epitope. The 3D structure was obtained from SWISS-MODEL (homology-modelling server), accessible via the ExPASy web server. The H protein of PPRV Nigeria 75/1 as a homo-dimer structure was constructed utilising the template crystal structure of measles virus H protein (Hashiguchi et al., 2007), Protein Data Bank identification code is 2zb5.1.A. Critical amino acid residues for C77 mAb binding site Y540 I542 Y543 are coloured in red and R547 S549 S550 in blue on the 3D surface of the H protein of PPRV Nigeria 75/1.

4.2.3 Construction of helper plasmids of PPRV Nigeria 75/1

The helper plasmids required for rescue of recombinant viruses, PPRV Nigeria 75/1 N (pGEM-N), P (pGEM-P) and L (pGEM-L), were cloned under the control of the T7 RNA polymerase promoter in the pGEM3z vector (Promega). The N, P and L coding sequences of PPRV Nigeria 75/1 were amplified (Figure 4.4) using gene specific primer sets (Appendix III) and pPPRV+GFP Nigeria 75/1 as the template. The amplicons were gel purified and digested with unique restriction enzymes compatible with the multiple cloning site in the pGEM 3z vector. The amplified N fragment was digested with *EcoR*I and *Sal*I enzymes, the P fragment was digested with *Sal*I and *Pst*I and the L fragment was digested with *Sal*I. The digested products were gel purified and ligated with the pGEM 3z vector separately that was prepared by digesting the vector with the corresponding enzymes. JM109 cells were transformed with the ligated products and any resulting colonies were screened by miniprep and restriction enzyme digestion. The plasmids (Figure 4.4) were sequenced on both the strands to ensure no unwanted nucleotide changes were present.

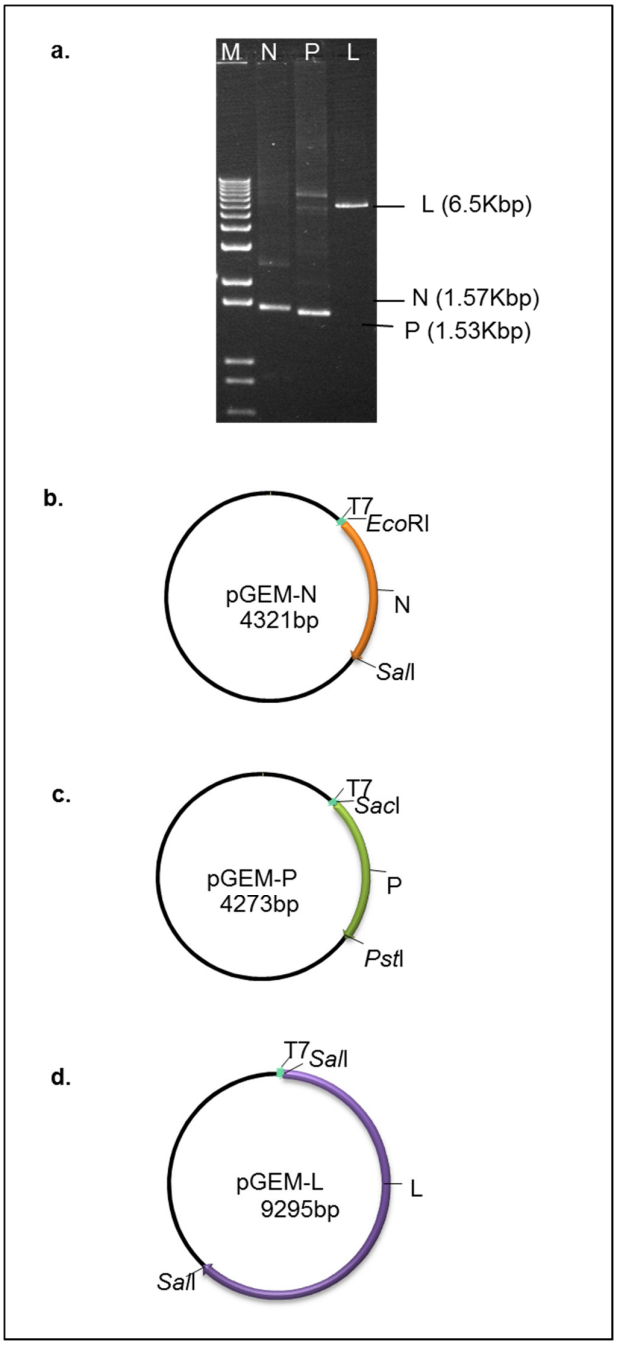

Figure 4.4 Construction of helper plasmids containing the N, P and L coding sequences of PPRV Nigeria 75/1. (a) amplification of N, P and L coding sequence of PPRV Nigeria 75/1 with fragment size indicated in parenthesis, M indicates 1Kb plus DNA ladder (Appendix II). (b to d) plasmids pGEM-N, pGEM-P and pGEM-L containing the N, P and L coding sequences of PPRV Nigeria 75/1, respectively. The N, P and L genes were cloned under the control of the T7 promoter using unique restriction enzyme sites as indicated.

4.2.4 Transfection and recovery of recombinant PPRV from full length genome plasmids

VDS cells were plated at $2.5x10^5$ cells per well in 6-well cell culture plate (BD Falcon) and incubated at 37°C and 5% CO_2 overnight. Cells (70% confluent) were washed with 1 ml of OPTI-MEM I reduced serum medium (Gibco) and infected with T7-polymerase expressing rFP virus at a MOI of 0.2 in 500 µl volume as described previously (Das *et al.*, 2000). The plates were incubated at 37°C and 5% CO_2 for an hour and rocked every 10 minutes for uniform distribution of the virus inoculum. During this period, the plasmid DNAs, TransFast Transfection Reagent (Promega) and OPTI-MEM I mix was prepared in order. Cells were washed and transfected with 1 µg of full-length PPRV cDNA plasmid and 1 µg pGEM-N, 1 µg pGEM-P and 0.05 µg pGEM-L using TransFast transfection reagent (Promega) at a ratio of 6:1 (wt/wt) in a total volume of 0.75 ml of OPTI-MEM I reduced serum medium/well (Gibco). Media was changed on cells at 24 hour post-transfection and observed for CPE for 3 days. Rescued viruses were harvested by freeze-thawing and further passaged in VDS cells.

4.2.5 Immunofluorescence and confocal microscopy

Immunofluorescences for the expression of N, H and or eGFP by the rescued viruses was carried out by labelling the N protein with an anti-PPRV-N C11 mAb and the H protein with anti-PPRV-H C77 mAb (BDSL, UK) and GFP autofluorescence (Mahapatra *et al.*, 2006; Parida *et al.*, 2007). Briefly, VDS cells were cultured on sterile glass coverslips in a 12-well plate to reach 60- 70% confluence and were infected with recombinant or wild PPRV Nigeria 75/1 viruses at an MOI of 0.01. Cells were fixed at 24 hours post-infection using 4% paraformaldehyde (PFA)

solution. The residual PFA was removed by washing the fixed cells twice with Ca/Mg free PBS. The cells were permeabilized using 0.1% Triton-X100 solution (Sigma) for 15 minutes and washed once with Ca/Mg free PBS. To block non-specific binding of antibodies in the downstream steps, permeabilized cells were treated with 0.5% bovine serum albumin solution prepared in Ca/Mg free PBS (BSA-PBS, Sigma) for 30 minutes. The blocked cells were treated with primary mAbs anti-PPRV-N C11 (1 in 10) or anti-PPRV-H C77 (1 in 40) for an hour. The C11 mAb was raised in mice at Indian Immunologicals, Hyderabad, India and the C77 was generated from a hybridoma at the Pirbright Institute and is commercially available (BDSL, UK). The cells were washed thrice with Ca/Mg free PBS to remove any excess unbound primary antibodies. The conjugated secondary antibody IgG Alexa Fluor 568 (Molecular probes) (diluted 1 in 200) was incubated on cells for one hour. The cells were washed trice with Ca/Mg free PBS and treated with 4',6 diamidino-2-phenylindole (DAPI) for 10 minutes to stain the cell nuclear DNA. The coverslips were washed with deionised water and mounted on glass slides using aqueous mounting medium (Vectasheild H1000). Any excess of mounting media was removed and the edges of the coverslips were sealed using a transparent nail varnish. The slides were viewed under the Leica TCS SP2 Acousto-Optical Beam Splitter confocal scanning laser microscope at an appropriate excitation wavelength of 405 ηm (Blue) 488ηm (Green) or 568ηm (Red). Expression of GFP was visualised by autofluorescence. Images were captured and processed with the Leica Confocal Software (Leica Microsystems).

4.3 Results

4.3.1 Rescue of recombinant PPRVs from cDNA clones

Infectious recombinant viruses of PPRV Nigeria 75/1, with and without the eGFP (rPPRV+GFP Nigeria 75/1 and rPPRV Nigeria 75/1, respectively) and rPPRV-C77c Nigeria 75/1 were rescued from respective cDNAs. In contrast, despite several attempts at virus rescue of live virus from pPPRV-C77a Nigeria 75/1 and pPPRV-C77b Nigeria 75/1 was unsuccessful. The CPE characteristic of PPRV infection was observed in the rescued viruses from three days post transfection with 100% efficiency. The CPE observed under light microscope for all the three recombinant viruses appeared to be identical to that of produced by the parental PPRV Nigeria 75/1 vaccine strain.

4.3.2 Confirmation of the identity of the rescued viruses

Total RNA isolated from the recovered PPRV recombinants (rPPRV+GFP Nigeria 75/1, rPPRV Nigeria 75/1 and rPPRV-C77c Nigeria 75/1) at passage 3 were subjected to RT-PCR using PPRV genome specific primers using a −RT as a control for carry over DNA. The expected amplicon sizes were observed on an agarose gel (Figure 4.5) and sequences were 100% identical to each respective plasmid (data not shown).

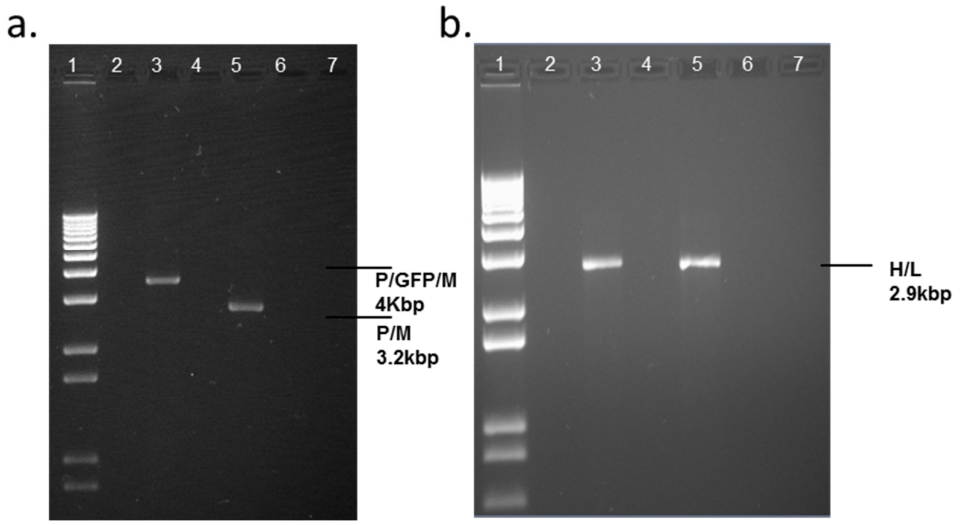

Figure 4.5 Confirmation of identity of rescued recombinant PPRVs. (a) RNA extracted from VDS cells infected with rPPRV+GFP Nigeria 75/1 or rPPRV Nigeria 75/1 were subjected to RT-PCR using specific primers sets (PPR-F7 and PPR-R10) located on the P and M gene of PPRV. Lane 1; Marker 1Kb plus (Appendix II), lane 2; PCR without RT enzyme with rPPRV+GFP Nigeria 75/1 RNA, lane 3; RT-PCR for rPPRV+GFP Nigeria 75/1 RNA, lane 4; PCR without RT enzyme with rPPRV Nigeria 75/1 RNA, lane 5; RT-PCR for rPPRV Nigeria 75/1 RNA and lane 6 (PCR) and 7 (RT-PCR) from VDS cell control RNA. (b) RNA extracted from VDS cells infected with rPPRV-C77c Nigeria 75/1 was subjected to RT-PCR using specific primers sets (PPR-F-15 and PPR-R-19) located on H and L gene of PPRV.

4.3.3 Immunofluorescence analysis for the assessment of eGFP expression and C77 mAb binding activity

Immunofluorescence imaging demonstrated the expression of the N and H proteins of PPRV in infected cells following labelling with specific mAbs and their expression was comparable to that observed with the commercially available vaccine virus (Figure 4.6). The rPPRV-C77c Nigeria 75/1 could not be detected by the anti-PPRV H mAb, but the viral N protein could be visualised using the anti-PPRV N mAb (Figure 4.6). The successful expression of eGFP from an additional transcriptional unit inserted within the PPRV genome was observed through its autofluorescence.

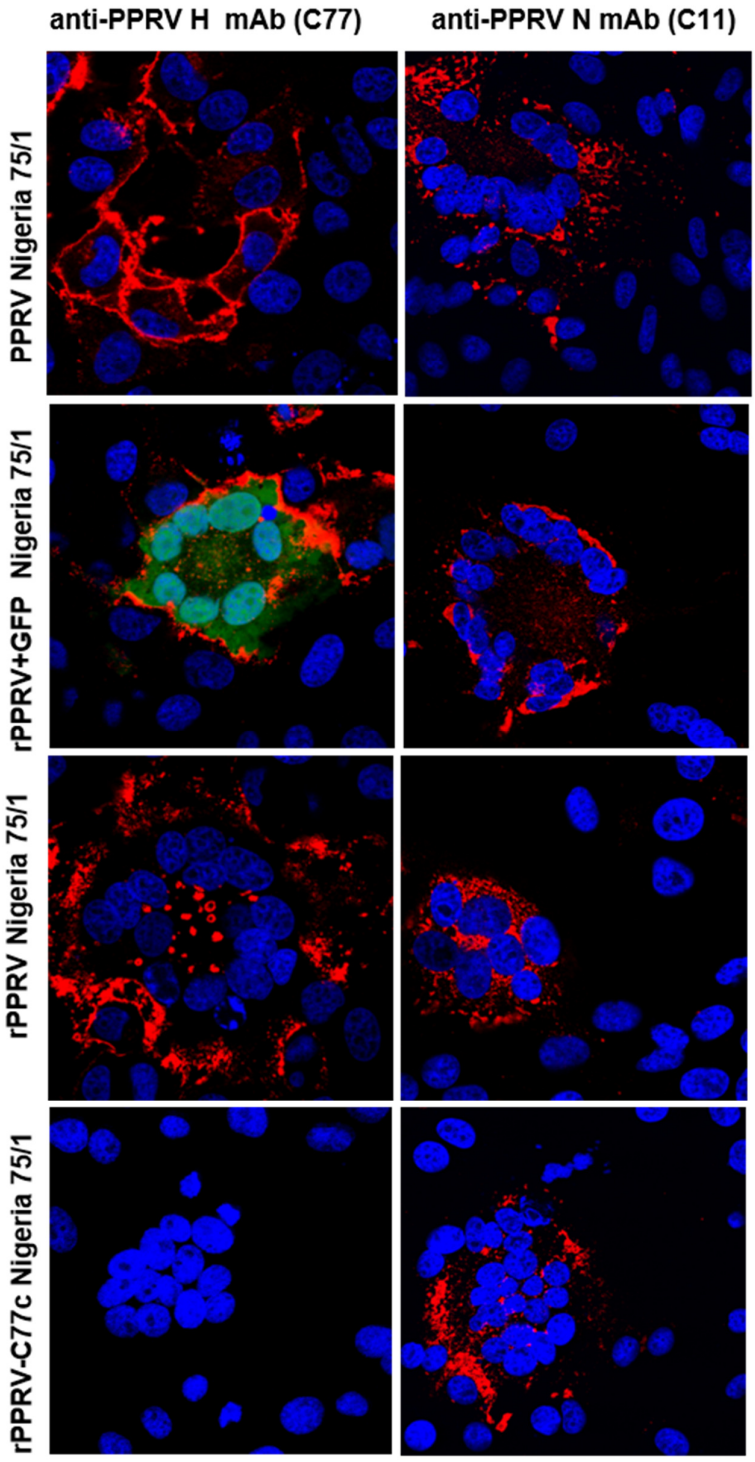
anti-PPRV H mAb (C77)
anti-PPRV N mAb (C11)
PPRV Nigeria 75/1
rPPRV+GFP Nigeria 75/1
rPPRV Nigeria 75/1
rPPRV-C77c Nigeria 75/1

Figure 4.6 Characterisation of rescued PPR viruses using confocal microscopy. Expression of PPRV N, and H proteins and/or GFP with C77 mAb binding activity in the recombinants and parental PPR viruses in infected cells. VDS cells were infected with viruses at an MOI of 0.01 and fixed 24 hours post-infection using 4% PFA. Cells were stained separately with primary antibodies of mouse anti-PPRV H (C77) and mouse anti-PPRV N (C11) followed by a secondary Alexa Fluor 568 goat anti-mouse antibody. Cell nuclei were stained with DAPI. Confocal laser scanning overlay of recombinant or parental PPRV infected VDS cells using three different wavelengths – blue (405 ηm) for cell nucleus, red (568 ηm) for H or N protein of PPRV and green (488 ηm) for GFP are shown. The expression pattern of PPRV N and H proteins were comparable between the recombinant and parental viruses. Wild-type H protein in PPRV Nigeria 75/1, rPPRV+GFP Nigeria 75/1 and rPPRV Nigeria 75/1 were detected using the C77 mAb whilst H protein was not detected using this antibody in cells infected with rPPRV-C77c Nigeria 75/1. The autofluorescence of GFP was detected in the green channel for the rPPRV+GFP Nigeria 75/1 virus labelled with anti-PPRV H mAb. Fluorescence at 488 ηm was not visualised for the image showing the rPPRV+GFP Nigeria 75/1 virus labelled with anti-PPRV N mAb.

4.3.4 *In vitro* growth characterization of recombinant PPRVs

The *in vitro* growth kinetics of the recombinant PPRVs and the parental virus was assessed in a multiple-step growth cycle as described (chapter 2). Multi-step growth curves were carried out to compare the growth of the recombinant viruses (rPPRV+GFP Nigeria 75/1, rPPRV Nigeria 75/1 and rPPRV-C77c Nigeria 75/1) with that of the parental vaccine strain (Nigeria 75/1) (Figure 4.7). The recombinant PPRVs grew to a similar titre and rate to that of the parental PPRV Nigeria 75/1 virus.

4.3.5 Assessing the stability of the heterologous protein and mutations to the C77 binding epitope

To determine the stability of the inserted eGFP transcriptional unit and the mutations made to the proposed C77 mAb binding epitope in the recombinant virus genome, viruses were serially passaged in VDS cells for up to 9 passages and assessed for the expression of GFP and maintenance of the C77 binding site associated mutations using RT-PCR followed by sequencing and confocal microscopy. No changes were found in the passaged viruses compared to the originally rescued viruses.

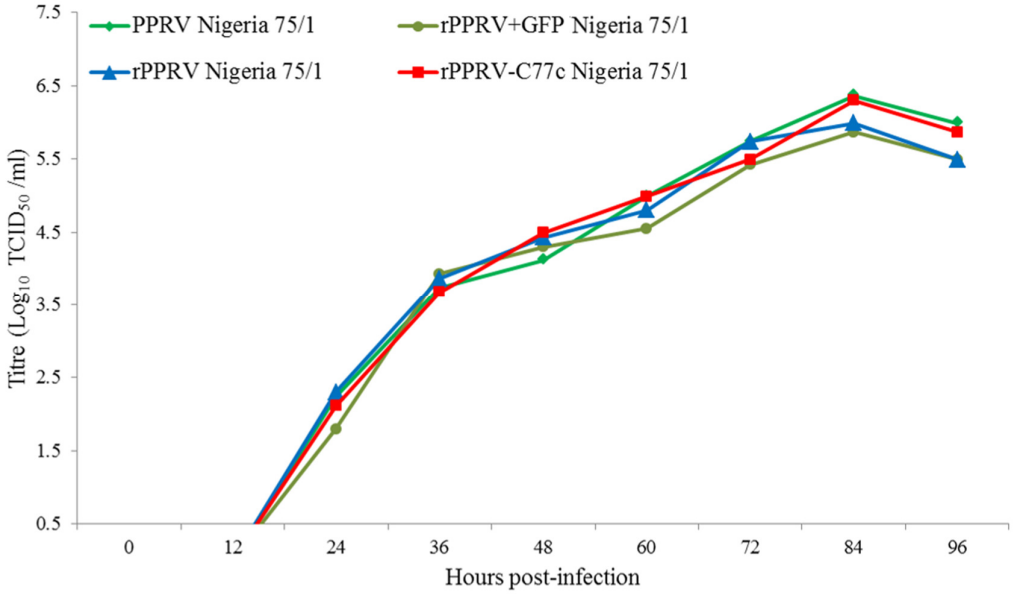

Figure 4.7 The growth kinetics of recombinant and parental PPRVs in cell culture. A multi-step growth curve was obtained by infecting VDS cells with virus at an MOI of 0.01 and assessing viral growth at different time points by virus titration ($TCID_{50}$).

4.4 Discussion

Despite the availability of reverse genetics techniques for other morbilliviruses, a rescue system for PPRV was lacking until successful rescue was reported almost simultaneously by Hu *et al* and Muniraju *et al* (Hu *et al.*, 2012a; Muniraju *et al.*, 2012; Muniraju *et al.*, 2015). Initial attempts to rescue a field isolate of PPRV were unsuccessful and following extensive investigation, the high GC rich region of the genome (between M and F ORF) was believed to be a potential bottleneck for viable virus rescue (Bailey, 2006). To overcome potential sequence errors introduced by the techniques involved in stitching together a full-length DNA copy of the viral genome, a synthetic DNA approach was applied to generate error free plasmids for virus rescue.

The insertion of unique restriction sites across the genome facilitates easy swapping of genes between PPRV isolates or from related viruses. The presence of unique restriction sites in RPV cDNA clone (Baron and Barrett, 1997) had facilitated swapping of M, F, H and N genes of PPRV efficiently (Das *et al.*, 2000; Mahapatra *et al.*, 2006; Parida *et al.*, 2007). The presence of unique non-viral restriction sites in RPV has not affected the potency of the rinderpest vaccine as they are located in the non-conserved parts of the UTRs of genome and did not alter the protein coding sequence of the ORFs (Das *et al.*, 2000; Mahapatra *et al.*, 2006; Parida *et al.*, 2007). The insertion of a novel transcription cassette within the genome facilitated ease of virus rescue and potential future evaluation of positive marker genes for DIVA activity. The new transcriptional unit was positioned between the P and the M genes of PPRV to ensure maintenance in the ratios of N and P protein required for efficient encapsidation, transcription and replication of the genomic RNA. In this study, the

insertion of eGFP within this transcription cassette is shown to be tolerated and facilitates potential future evaluation of positive marker genes for DIVA activity. Similar approaches had been investigated previously for RPV where *in vitro* development of positively and negatively marked vaccines has been assessed (Brown *et al.*, 2005; Buczkowski *et al.*, 2012; Das *et al.*, 2000; Mahapatra *et al.*, 2006; Parida *et al.*, 2007; Walsh *et al.*, 2000a; Walsh *et al.*, 2000b). Following the rescue and passage of the recombinant PPRV, CPE and growth characteristics observed with the recombinant virus was similar to that of the parental virus as seen by Hu et al (Hu *et al.*, 2012a). The cellular distribution of N and H proteins in the recombinant virus was the same as in cells infected with the parental virus and was as expected for PPRV.

Out of the three H mutated full-length c-DNAs (pPPRV-C77a, pPPRV-C77b and pPPRV-C77c), only the pPPRV-C77c was successfully rescued. The mutation of residues (R547 S549 S550) in pPPRV-C77c within H demonstrated the potential for a negative marker vaccine and H gene functionality and stability of the introduced mutations. A predicted 3D structure of the H protein of PPRV modelled using the crystal structure of measles virus H protein indicated that the amino acids Y540 I542 Y543 were buried in the C77 mAb binding site and residues R547, S549, and S550 of H protein are present on the outer surface. Live infectious virus could not be rescued from plasmids (pPPRV-C77a and pPPRV-C77b) containing substitutions at Y540 I542 Y543. Mutation of the pPPRV Nigeria 75/1 plasmid at residues R547 S549 S550 allowed virus to be rescued suggesting that the Y540 I542 Y543 amino acids could be involved in important biological functions necessary for H protein function. These amino acids Y540 I542 Y543 region are highly conserved among all

the known PPRV isolates, but not the R547 S549 S550 region (Sungri 1996 vaccine strain has a leucine at position 547). However, it is possible that one or more amino acid in this region could be critical for the viability of the virus. Therefore, it would be interesting to find out whether single or combination of two amino acids substitution in this region (Y540 I542 Y543) would lead to a successful recovery of viable virus.

The reverse genetics system developed in this study could be utilised for the investigation of the basic molecular biology of PPRV including protein-protein interactions, host-pathogen interactions or as a potential vaccine vector to express foreign immunogens to make a multivalent vaccine. The rPPRV-C77c Nigeria 75/1 virus has been further assessed *in vivo* (Muniraju *et al.*, 2015) (Chapter 5) for its vaccine potency, safety and DIVA strategy for use in the field that has the potential for serological differentiation between vaccinated and infected animals during a disease control programme.

Chapter 5 *In vivo* evaluation of the rPPRV-C77c Nigeria 75/1 rescued vaccine virus and its comparison with the parent PPRV Nigeria 75/1 vaccine strain

5.1 Introduction

In Chapter 4, the construction and rescue of various recombinant full-length PPRVs (rPPRV+GFP Nigeria 75/1, rPPRV Nigeria 75/1 and rPPRV-C77c Nigeria 75/1) and their *in vitro* characterisation was described. All the recombinant viruses were shown to be similar to their parental virus (PPRV Nigeria 75/1) in terms of CPE and syncytia formation and had comparable growth characteristics (rate and titre). The mutated H protein of rPPRV-C77c Nigeria 75/1 virus did not bind to the C77 mAb in VDS cells in an immunofluorescence study. Therefore, the next aim was to evaluate the rPPRV-C77c Nigeria 75/1 virus *in vivo* to determine its safety and potency and its fitness as a DIVA vaccine to differentiate between infected and vaccinated animals. As the mutation to the H protein had rendered the C77 mAb unable to bind to H protein *in vitro* we hypothesised that the serological response to the mutated H protein in vaccinated goats would be unable to bind to the H protein antigen present coated on the plates in the c-H ELISA (BDSL, UK). We also hypothesised that the serological response to the mutated H protein would enable differentiation between sera from vaccinated and naturally infected animals.

5.2 Materials and methods

5.2.1 Viruses

The recombinant virus rPPRV-C77c Nigeria 75/1 and the parental PPRV Nigeria 75/1 vaccine viruses were used for vaccination. The virulent PPRV Ivory Coast 89/1

was used as challenge virus. The rescued rPPRV-C77c Nigeria 75/1 (passage 3) and PPRV Nigeria 75/1 were grown in VDS cells to attain higher titres of $TCID_{50}$ (assessed in VDS cells). The PPRV Ivory Coast 89/1 strain, a virulent field strain, was passaged once in lamb kidney cells, before storage at -80 °C (Herbert *et al.*, 2014; Mahapatra *et al.*, 2006).

5.2.2 Animal experiment design

Animal experimentation was conducted according to UK Home Office regulations (Project licence number: 70/6907) and following ethical approval from The Pirbright Institute, UK. European mixed breeds of 12 male goats, aged 6 to 9 months were randomly split into three groups (n= 4/ group) as shown in Table 5.1. Goats were kept under observation for a week for acclimatisation in the isolation unit at the Pirbright Institute. Animals in group one (G1, G2, G3 and G4) were immunised with 10^4 $TCID_{50}$ rPPRV-C77c Nigeria 75/1 virus whilst animals in group two (G7, G8, G9 and G10) received the PPRV Nigeria 75/1 conventional vaccine virus (10^4 $TCID_{50}$) via the sub-cutaneous route in a total volume of 1 ml (diluted in PBS). Groups one and two were housed in separate rooms along with two unvaccinated control animals per room (G5 and G6 with group one and G11 and G12 with group two). Animals were monitored daily for 28 days post vaccination (dpv). At 4 weeks post vaccination, all 4 control goats were segregated into a separate room and the animals from all 3 treatment groups were challenged with a pathogenic PPRV Ivory Coast 89/1 isolate (10^5 $TCID^{50}$) by the intranasal route using a LMA® MAD Nasal™ Intranasal Mucosal Atomization Device (LMA, San Diego, USA). Animals which developed severe clinical signs were humanely terminated according to an established clinical score-card (Pope *et al.*, 2013). Killing of animals was performed

by intravenous administration of a lethal dose of pentobarbitone (Vetoquinol, France).

Table 5.1 Animal experiment design and treatments

Animal group	Animal number	Vaccine treatment
Group 1	Goat 1 (G1)	rPPRV-C77c Nigeria 75/1 vaccine
	Goat 2 (G2)	
	Goat 3 (G3)	
	Goat 4 (G4)	
Group 2	Goat 7 (G7)	PPRV Nigeria 75/1 vaccine
	Goat 8 (G8)	
	Goat 9 (G9)	
	Goat 10 (G10)	
Group 3	Goat 5 (G5)	Unvaccinated control
	Goat 6 (G6)	
	Goat 11 (G11)	
	Goat 12 (G12)	

5.2.3 Collection of clinical samples

For all animals, rectal temperatures and clinical assessments were conducted twice daily. Ocular, nasal and saliva swabs (Table 5.2) were taken in 0.5 ml lysis buffer (MagNA Pure Roche, UK) on every alternate day for the first 2 weeks of both the vaccination and challenge period and then at weekly intervals to analyse the presence of PPRV nucleic acid by real-time RT-PCR using PPRV N gene specific primers (Batten *et al.*, 2011). For virus isolation, samples were collected in 1 ml of PBS and used to infect VDS cells in a 25cm^2 flask. Heparinised blood samples (Table 5.2) were collected for virus isolation from peripheral blood mononuclear cells (PBMCs) by co-cultivation with VDS cells. Clotted blood samples were collected for the evaluation of serum antibody responses specific to the PPRV H protein using a PPR Antibody ELISA kit (BDSL, UK) and the presence of PPRV neutralising antibodies were assessed using a virus neutralisation test (VNT).

Table 5.2 Clinical sample collection schedule

Vaccination / challenge period	Days post-vaccination (dpv)/ post-challenge (dpc)	Sample name
Vaccination	0 dpv	Heparinised blood Clotted blood Ocular swab Nasal swab Saliva swab
	2 dpv	
	4 dpv	
	6 dpv	
	8 dpv	
	10 dpv	
	12 dpv	
	15 dpv	
	22 dpv	
Challenge	28 dpv / 0 dpc	Heparinised blood Clotted blood Ocular swab Nasal swab Saliva swab
	2 dpc	
	4 dpc	
	6 dpc	
	8 dpc	
	10 dpc	
	12 dpc	
	14 dpc	

5.2.4 White blood cells (WBCs) counts

Heparinised blood was diluted 1:20 with 0.174 M acetic acid (10 µl blood + 190 µl glacial acetic acid) to lyse the red blood cells (RBC). Leucocytes were then counted using a modified Fuch's- Rosenthal haemocytometer and the cell count was expressed as the total number of leucocytes per mm^3 of blood.

5.2.5 Whole blood FACS analysis of lymphocytes

The change in the proportion of live lymphocyte subsets ($CD4^+$, $CD8^+$, $CD14^+$ and WC1/ γδ T cells) in peripheral blood samples collected from goats during both the vaccination and challenge period was analysed by antibody staining and flow cytometry as described by Herbert *et al* (Herbert *et al.*, 2014). Monoclonal antibodies against cell surface markers used included $CD4^+$ (647 clone 44.38, MCA2213A647 from Serotec), $CD8^+$ (575 clone CC63, MCA837 from Serotec), $CD14^+$ (405 clone CCG33 from Serotec) and WC1 (405 clone 197 from Serotec).

Appropriately diluted antibodies in a 25 µl volume along with 0.5 µl of live/dead aqua (495 life technologies) per sample was prepared and added in 200 µl of heparinised blood, vortexed and incubated for 20 minutes at room temperature. RBCs were lysed by adding 1 ml of RBC lysis buffer (Appendix I) and vortexed for 1 minute. The mixture was diluted with 5 ml of PBS and centrifuged at 280 g for 5 minutes. Cells were fixed in 200 µl of 4% PFA for 20 minutes. PFA was removed by diluting with 5 ml of PBS and centrifuged at 280 g for 5 minutes. Centrifuged cell pellets were re-suspended in 400 µl of PBS and read using the LSR Fortessa (BD Biosciences) with data acquired using the DIVA software (BD Biosciences) and analysed using FlowJo software (Tree Star Inc). Simultaneously, sample controls for live/ dead only and the single stain control with $CD4^+$, $CD8^+$, $CD14^+$ and WC1 containing live/dead aqua were prepared for compensation to correct the spectral overlap required in multicolour flow cytometry.

5.2.6 Separation of goat peripheral blood mononuclear cells

PBMCs were isolated from the heparinised peripheral blood samples using histopaque (Sigma 1083) density gradient centrifugation. 15 ml blood was diluted with an equal volume of PBS and 15 ml of histopaque (1.083 g/ml) was under-laid using a pipette. The samples were centrifuged for 45 minutes at 280 g at 20°C with the brake off. The buffy coat was carefully aspirated and residual RBCs were lysed using an ammonium chloride RBC lysis buffer (Appendix I). Cells were washed twice with 30 ml of ice-cold PBS and centrifuged for 10 minutes at 280 g at 4°C. The final cell pellet was re-suspended in 2 ml of complete media and cells were counted under the light microscope by staining the cells with a solution of trypan blue (0.4%, Gibco).

5.2.7 Virus isolation from the eye swabs and PBMCs

Ocular swabs collected in PBS were squeezed on the wall of the tube and the released fluid was added to VDS cells (60-70% confluent) in 25 cm^2 flask. The flasks were incubated at 37°C for an hour to allow virus adsorption with intermittent rocking of each flask every 15 minutes to ensure a uniform distribution of the inoculum. To the flasks, 4 ml of media (DMEM with 2.5% FCS and penicillin [100 U/ml, Sigma]/ streptomycin sulphate [100 µg/ml, Sigma] and amphotericin B [2.5 µg/ml, Sigma]) was added and incubated at 37°C for 5 days and observed under light microscope for assessment of CPE formation. Three blind passages were performed to ensure detection of low levels of virus.

For virus isolation from PMBCs, fresh goat PBMCs (~1 x 10^6 cells in 1 ml total volume) were added to VDS cells (60-70% confluent) in a 25 cm^2 flask containing 5 ml of DMEM (2.5% FCS and penicillin / streptomycin sulphate and amphotericin) and incubated at 37°C overnight. The following day the PBMC were removed by washing the cell layer twice with PBS, the media was replaced and the cells were incubated at 37°C for another 4 days and observed under light microscope for assessment of CPE formation. As with attempts at virus isolation from ocular swabs, three blind passages were performed to maximise the potential for isolation.

5.2.8 Virus neutralisation test

The VNT was performed using VDS cells by following the method as described previously (OIE, 2012). Briefly, in 96-well microtitre cell culture plates, heat inactivated sera (at 56 °C for 30 minutes) was serially diluted two-fold in DMEM across the plate (100 µl per well and 8 replicates per dilution). An aliquot of PPRV

(100 µl containing 100 $TCID_{50}$) was added to all wells including the virus control wells (no serum), with the exception of the cell control. The plates were incubated for an hour at 37 °C to allow serum virus reaction before being to 50 µl of VDS cells (1 x 10^5 cells per ml). The plates were incubated at 37 °C/ 5% CO_2 for 7 days and read under light microscope to evaluate the presence/absence of CPE. In parallel, the virus used for the assay was titrated to re-confirm the anticipated titre (2000 to 2500 $TCID_{50}$ per ml). The virus titre was calculated according to the methods of Reed & Muench (1938) and the virus serum neutralisation titres were expressed as log_{10} with titres greater than 3 being considered positive.

5.2.9 Competitive H protein ELISA

The PPRV H protein-specific antibody response in the serum of vaccinated and challenged animals was assessed using the c-H ELISA (BDSL, UK). The assay works on the principle that antibodies to the PPRV H protein in test sera compete with the mAb (C77) in the test kit for binding to PPRV antigen. The binding of the C77 mAb is then detected using a secondary anti-mouse IgG conjugated to horseradish peroxidase and ortho-phenylene diamine (OPD) as substrate/chromogen. Briefly, the 96-well microtitre plate was coated with 50 µl of inactivated whole virus antigen appropriately diluted in PBS and incubated for an hour at 37°C on an orbital shaker. Following antigen adsorption, the plates were washed 3 times using PBS. Test sera were added at a dilution of 1/5 in a total volume of 50 µl blocking buffer (0.1% [v/v] Tween-20 and 0.3% [v/v] normal bovine serum) followed immediately by the addition of the C77 mAb at a dilution recommended by the manufacturer. Serum, antigen and mAb controls were included as recommended by the manufacturer. Following incubation for an hour at 37°C on an orbital shaker, plates

were washed thrice and incubated with secondary anti-mouse IgG conjugated to horseradish peroxidase for an hour at 37°C on an orbital shaker. Plates were washed thrice and the OPD substrate was added for 10 minutes and the plates were read using a VMax Kinetic ELISA microplate reader (Molecular devices) at an absorbance of 492 ηm. The optical density (OD) values were converted to percent inhibition (PI) values using the formula below:

$$PI=100-([OD \text{ in test well}/OD \text{ in mAb control well}] \times 100)$$

Percentage inhibition values greater than 50% were considered positive as per the manufacturer's instructions.

5.2.10 RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

For viral RNA isolation swabs were collected in 0.5 ml of lysis buffer (MagNA Pure kit) and stored at -20° C until required for processing. RNA extraction was achieved using robotic extraction methods (MagNA Pure LC Total Nucleic Acid Isolation Kit, Roche, UK) following the manufacturer's protocols.

The qPCR was performed following the method as described by Batten *et al* (Batten *et al.*, 2011). Briefly, the RT-PCR reaction was performed using the Superscript III/Platinum Taq one-step RT-PCR kit (Invitrogen, Paisley, UK) using the Stratagene Mx3005p real-time PCR machine. Primers, probe and reaction condition were considered as described by Batten *et al* (Batten *et al.*, 2011) with minor modifications. Briefly, 6 µl of test sample positive and negative control RNA were

loaded in duplicate onto 96-well optical reaction plates (Stratagene, UK). The PCR reaction mix of 19 µl was loaded into each well and contained 12.5µl Superscript III/ Platinum Taq One-step RT-PCR reaction mix, 0.5 µl Superscript III/ Platinum Taq One-step RT-PCR enzyme mix, 0.5 µl ROX reference dye, 0.5 µl probe (5 pmol/ µl), 1 µl each of the forward and reverse primers (10 pmol/ µl) and 3µl nuclease free water.

The thermal cycling conditions were as follows:

Step 1: Reverse transcription (RT)		50°C	30 minutes
Step 2: RT inactivation and DNA polymerase activation		95°C	10 minutes
Step 3:	Denaturation	95°C	15 seconds
Step 4:	annealing and elongation	60°C	1 minutes
Step 5: Steps 3 and 4 repeated for 45 cycles			

Stratagene MxPro software (Stratagene, USA) was used for the data analysis. The threshold fluorescence was set using the software algorithm amplification-based threshold method. Samples with no detectable fluorescence above the threshold after 40 cycles were considered negative (Batten *et al.*, 2011).

5.2.11 Sequencing of the C77 mAb binding epitope

The viruses isolated from the PBMCs of vaccinated animals were sequenced using a specific primer set PPR-F-P18 and PPR-R-P18 (Appendix III) based on the H gene of PPRV Nigeria 75/1.

5.3 Results

5.3.1 Clinical protection of goats vaccinated with either the rPPRV-C77c or the conventional PPR vaccine following challenge with a virulent strain of PPRV

Following vaccination, all animals remained healthy and did not show any vaccination-related adverse effects. Similarly, 28 dpv when the goats were challenged with virulent PPRV (Ivory Coast 89/1 strain), no clinical signs or high rectal temperatures (Figure 5.1) were observed in either the recombinant or conventional vaccinated groups. In contrast, the control unvaccinated animals developed severe clinical disease (pyrexia, mucopurulent nasal discharge, severe conjunctivitis, congested oro-nasal mucosa, lose motion and anorexic) and were humanely terminated at 8 days post challenge (dpc). The disease onset in control animals was observed from 3 dpc with an increase in rectal body temperatures to greater than 40°C. The increased temperatures remained high up to 8 dpc with the peak temperatures observed on 5 and 6 dpc. Animals in the vaccinated groups maintained their rectal body temperatures within the normal temperature range throughout the vaccination and challenge period of the experiment, displaying no clinical symptoms.

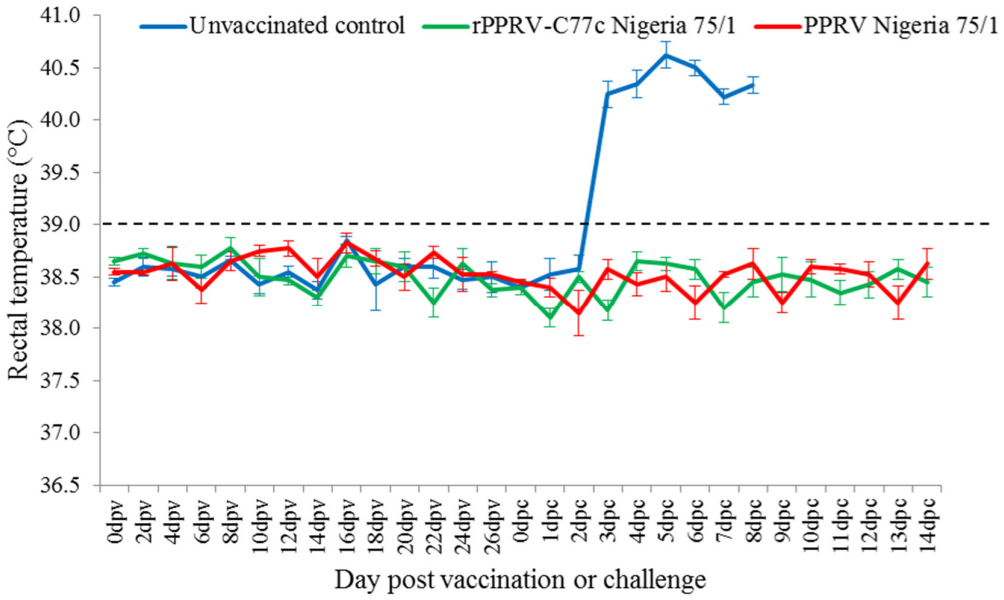

Figure 5.1 Rectal temperatures of vaccinated (rPPRV-C77c Nigeria 75/1 and PPRV Nigeria 75/1 parent vaccine virus) and unvaccinated goats upon challenge with virulent PPRV. Temperatures were measured twice per day, every alternate day during the vaccination period and every day during challenge period, and are presented as the mean values of the four animals in each group with the standard error. The threshold for pyrexic temperatures is indicated by the dashed line.

5.3.2 The immunosuppressive effect of PPRV on rPPRV-C77c Nigeria 75/1 and PPRV Nigeria 75/1 vaccinated animals upon challenge with virulent PPRV

Suppression of the immune system during PPRV infection is due to the lymphotropic nature of PPRV and the diseased animal often develops a pronounced leukopenia, facilitating secondary infection. Vaccination with rPPRV-C77c Nigeria 75/1 or the conventional live attenuated PPRV vaccine overcomes this immunosuppressive following natural infection with virulent PPRV and thus prevents exacerbation of opportunistic infection by secondary pathogens. No change in WBCs count was observed during the post-vaccination period in either of the vaccinated groups (Figure 5.2 and Appendix IV) except a transient reduction of WBC counts at 4 to 6 dpv. A significant ($P<0.0001$) reduction in live WBC counts was observed from 4 dpc to 8 dpc (the day of sacrifice of control goats on ethical grounds) in unvaccinated control animals in comparison to the WBC counts for both groups of vaccinated animals (Figure 5.2). No significant reduction ($p<0.0001$) in WBC counts was observed in either of the vaccinated groups during the challenge period. However, the leukocyte counts for both of the vaccinated groups went slightly down on 6 dpc and this transient reduction of leucocyte counts started increasing by 8 dpc.

Further, analyses of whole blood samples using FACS staining of samples taken during the post-vaccination and post-challenge periods demonstrated, a specific and significant (2way ANOVA $p<0.0001$) decrease in $CD4^+$ cells from day 4 post-challenge in the non-vaccinated control animals (Figure 5.3a). The $CD8^+$, $CD14^+$ and WC1 cell counts in both vaccinated and unvaccinated animals were not altered significantly ($P>0.05$) in comparison with the normal cell counts (Figure 5.3b to d).

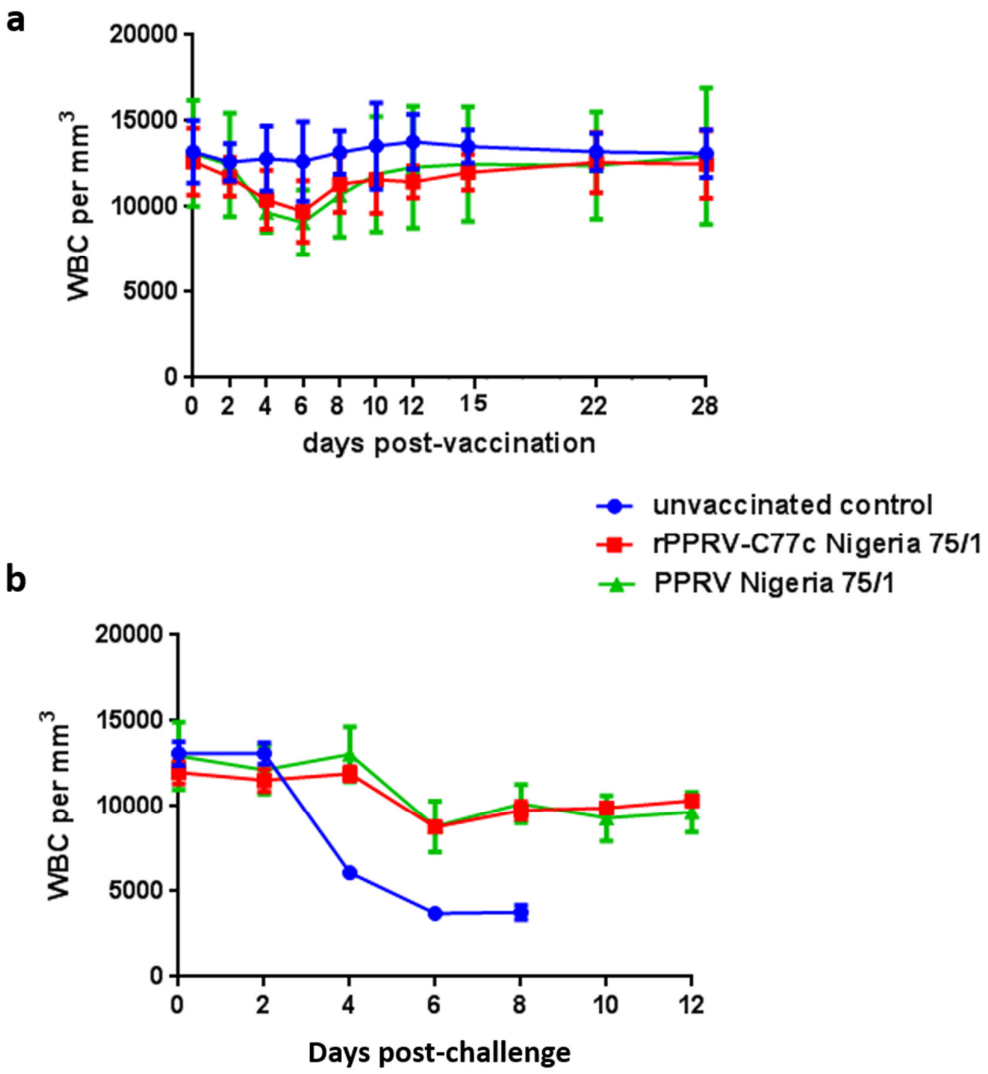

Figure 5.2 Leukocytes (WBC) counts for both vaccinated and unvaccinated goats following a virulent PPRV challenge. The total leukocytes were counted using a Neubauer haemocytometer and expressed as the total number of WBCs per ml of blood. The mean values of the four animals in each group ± the standard error of the mean are shown for both vaccination (a) and challenge periods (b).Significant differences in WBC counts between vaccinated and unvaccinated control groups (2way ANOVA $p<0.0001$) were observed from 4 days post-challenge.

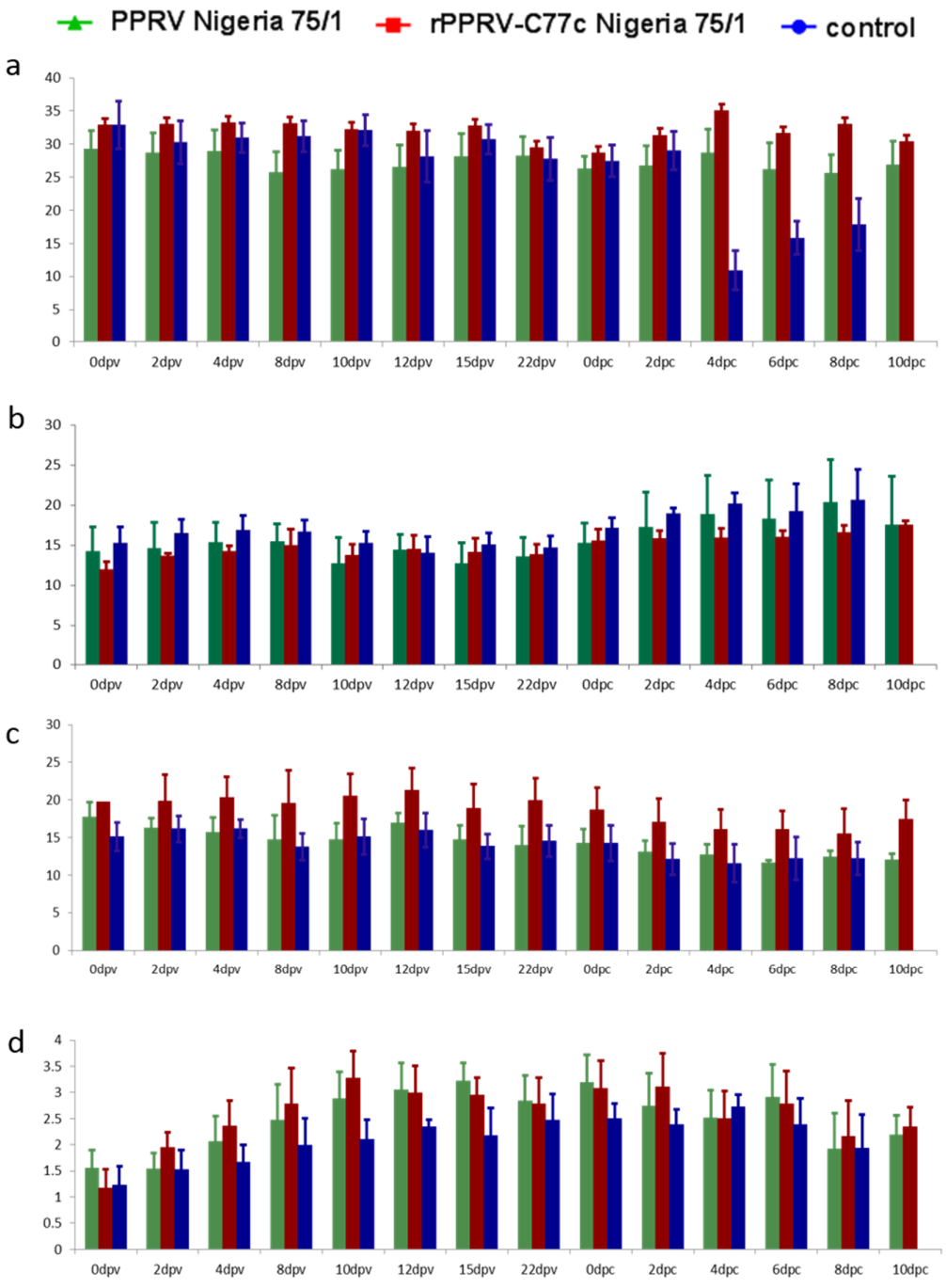
PPRV Nigeria 75/1
rPPRV-C77c Nigeria 75/1
control
a
b
c
d
0dpv
2dpv
4dpv
8dpv
10dpv
12dpv
15dpv
22dpv
0dpc
2dpc
4dpc
6dpc
8dpc
10dpc

Figure 5.3 $CD4^+$, $CD8^+$, WC1 T-cell and $CD14^+$ monocyte/macrophage cell counts in vaccinated and control goats by whole blood FACS analysis. Blood was stained with anti-$CD4^+$, anti-$CD8^+$, anti-WC1 and anti-$CD14^+$ monoclonal antibodies directly conjugated to flurochromes. Results were expressed as the mean percentage ± standard error of $CD4^+$ lymphocytes (a), $CD8^+$ lymphocytes (b), WC1 lymphocytes (c) and $CD14^+$ monocytes/macrophages (d) in peripheral blood. Significant differences in $CD4^+$ cells between vaccinated and unvaccinated control groups (2way ANOVA $p<0.0001$) were observed from 4 days post-challenge. Whole blood FACS analysis for 6 day post-vaccination had not been conducted.

5.3.3 Vaccination prevents the replication of challenge virus in vaccinated goats

Detection of virus specific RNA and live virus was observed in the eye swabs of control animals during the post-challenge period whereas no virus/ viral genome was detected from either of the vaccinated groups throughout the post-challenge period. To determine the level of virus replication and excretion in vaccinated and unvaccinated goats during the post-challenge period, clinical samples that had been collected from the eyes, nasal turbinates and saliva on each alternate day were subjected to extraction of RNA and qPCR. The actual Ct-values were subtracted from 40 (accepted maximum Ct value) and expressed as 40-Ct values. As shown in Figure 5.4, no Ct values were observed in vaccinated groups (rPPRV-C77c Nigeria 75/1 and PPRV Nigeria 75/1) throughout the challenge period with few exceptions in both the vaccinated groups, specifically in nasal and saliva samples of animals at 6 to 10 dpc (Appendix V). The unvaccinated control animals were positive by qPCR from 2 dpc in nasal and 4 dpc in ocular swabs and 6 dpc in all 3 samples (saliva, nasal and ocular). The Ct values in the unvaccinated control animals were high at 6 dpc for all clinical samples and reached peak values at 8 dpc. Although the viral genome was detected late in saliva samples (6 dpc), the highest copy number was obtained in saliva samples from 6 to 8 dpc.

Similarly, the ocular swab samples collected during the post-challenge period were used to isolate virus in VDS cells. It was not possible to recover virus from swabs taken from animals vaccinated with either rPPRV-C77c Nigeria 75/1 or PPRV Nigeria 75/1 following vaccination or challenge, even after three blind passages. However, it was possible to isolate PPRV successfully from the eye swabs of the unvaccinated control animals between 4 dpc and 8 dpc, the day of sacrifice (Table 5.3).

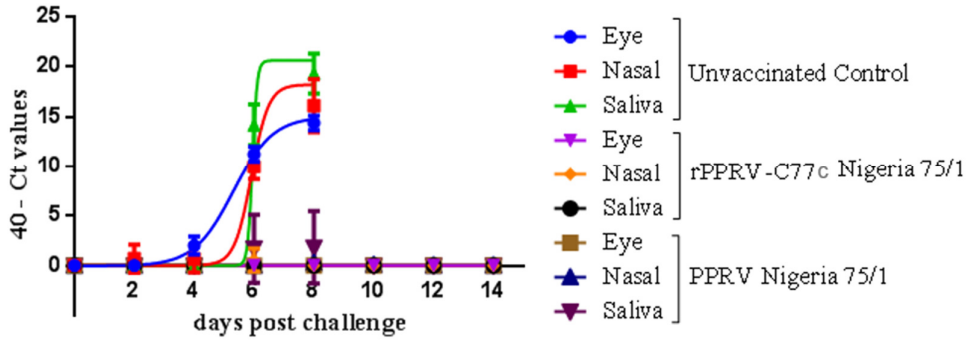

Figure 5.4 Real time RT-PCR analyses for PPRV in lachrymal, nasal and salivary excretions during the post-challenge period. RNA extracted from clinical swab samples collected every alternative day post-challenge from each of the vaccinated and control goats were subjected to real-time PCR using specific N gene primers of PPRV. The mean 40-Ct value of each group was presented with ± standard error mean. The mean 40-Ct values ranging from 1 - 3 are considered as weak positive, 3 - 12 are positive and more than 12 are strong positive.

Table 5.3 PPRV isolation from clinical eye swab sample during post-challenge period.

Treatment group	Animal number	Virus isolation*							
		0dpc	2dpc	4dpc	6dpc	8dpc	10dpc	12dpc	14dpc
rPPRV-C77c Nigeria 75/1 vaccine	G1	N	N	N	N	N	N	N	N
	G2	N	N	N	N	N	N	N	N
	G3	N	N	N	N	N	N	N	N
	G4	N	N	N	N	N	N	N	N
PPRV Nig75/1 vaccine	G7	N	N	N	N	N	N	N	N
	G8	N	N	N	N	N	N	N	N
	G9	N	N	N	N	N	N	N	N
	G10	N	N	N	N	N	N	N	N
Control un-vaccinated	G5	N	N	Y	Y	Y	NA	NA	NA
	G6	N	N	Y	Y	Y	NA	NA	NA
	G11	N	N	Y	Y	Y	NA	NA	NA
	G12	N	N	Y	Y	Y	NA	NA	NA

* 'N' represents no virus isolation, 'Y' represents virus isolation, 'NA' represents not applicable, dpc represents day post-challenge.

5.3.4 The mutations introduced to rPPRV-C77c Nigeria 75/1 were stably maintained in vaccinated animals

The recombinant virus isolated from the PBMCs of with rPPRV-C77c Nigeria 75/1 virus vaccinated goats at 4 dpv (Goat 1 and Goat 3) and 8 dpv (Goat 2 and Goat 4) confirmed that the mutated C77 mAb binding site on H protein of rPPRV-C77c had not been altered (Figure 5.5) or reverted back to its native sequence.

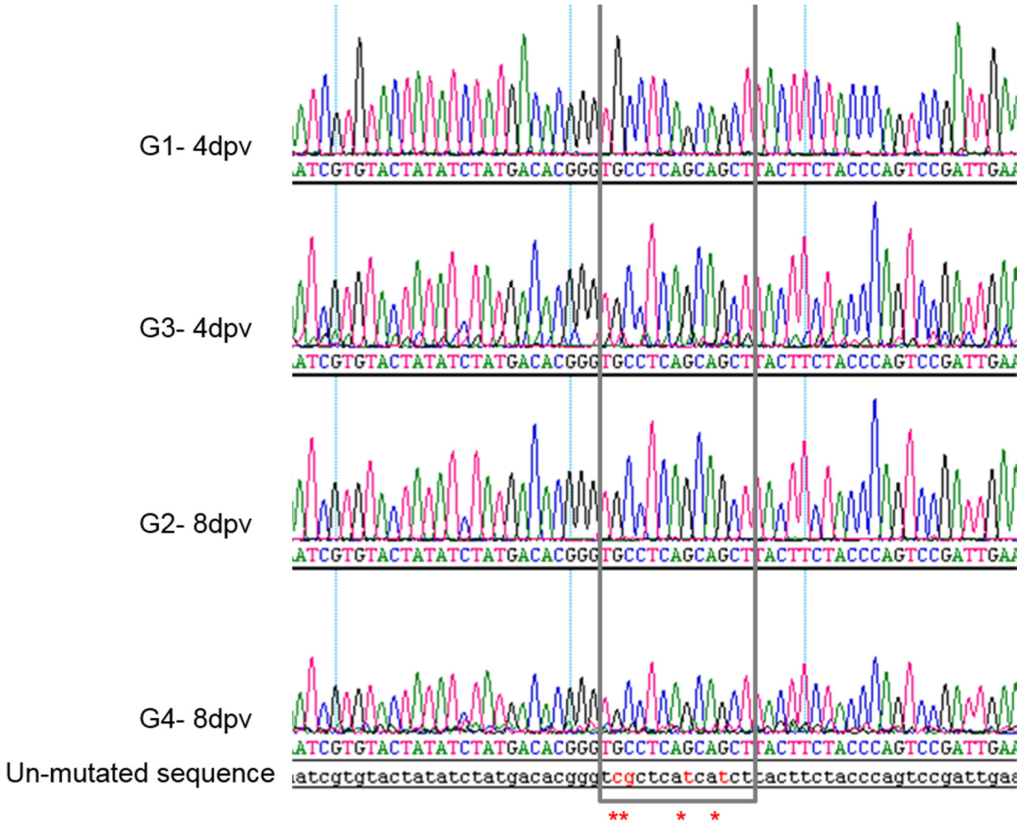

Figure 5.5 Sequence analysis of the rPPRV-C77c Nigeria 75/1 virus H protein following *in vivo* analysis. The mutations to the modified C77 mAb binding region were maintained following a passage of the mutated virus in goats. The RNA was extracted from virus isolated from PBMCs on 4 (from G1 and G3) and 8 (from G2 and G4) days post-vaccination. An alignment of nucleotide sequences of the C77 mAb binding region on the rPPRV-C77c Nigeria 75/1 virus with that of parental PPRV Nigeria 75/1 is shown. * indicates the residues mutated in at the proposed C77 binding site in the rPPRV-C77c Nigeria 75/1 virus.

5.3.5 rPPRV-C77c Nigeria 75/1 virus induces a similar level of PPRV specific neutralizing antibodies to that induced by the parent Nigeria 75/1 vaccine strain

Sera collected during both the vaccination and challenge periods were subjected to VNT using homologous PPRV vaccine strains. On the day of vaccination, animals were found to be negative for PPR specific antibodies whilst high titre virus neutralising antibodies were detected by second week of vaccination and reached at peak (4.76–5.31 $\log_{10}$) on 28 dpv, the day the animals were challenged, in both groups of vaccinated animals (Figure 5.6 and Appendix VI). All unvaccinated control animals remained negative during this vaccination period. Following challenge, titres of antibodies in both the vaccinated groups remained constant at two weeks post challenge. No statistically significant difference in neutralising antibody titre was seen between the recombinant and conventional vaccine groups. The unvaccinated animals developed PPRV specific neutralising antibodies by the 8 dpc, the day they were humanely terminated. Significant differences in the titer of neutralizing antibody was seen (2way ANOVA at $p<0.0001$) between the vaccinated groups (rPPRV-C77c Nigeria 75/1 and PPRV Nigeria 75/1) and the control groups at all days of sample analysis except on 0 dpv. However, no significant differences of neutralising titre were observed ($P > 0.05$) between both the vaccinated groups at all the sampling time points.

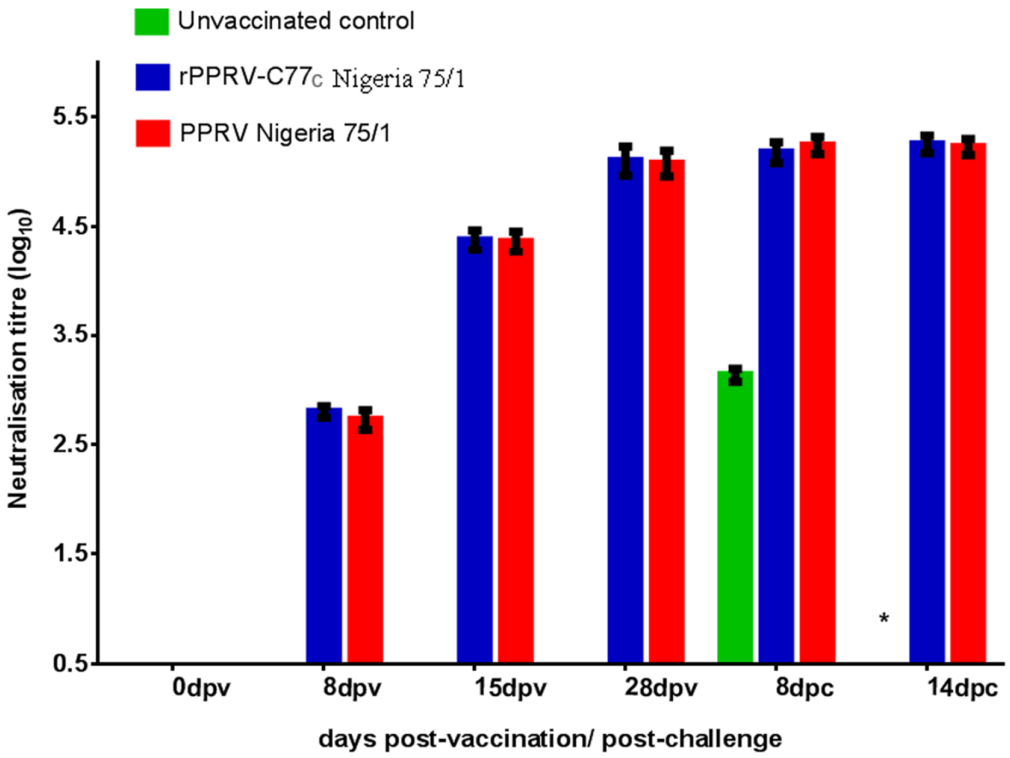

Figure 5.6 Detection of PPR specific neutralising antibodies in vaccinated and challenged goats. Mean virus neutralising titres (expressed as Log_{10}) in vaccinated and challenged goats as assessed by virus neutralisation test. Serum samples were analysed at 0, 8, 15 and 28 days post-vaccination and 8 and 14 days post-challenge. * Represents a sample omission from the control unvaccinated animals due to their sacrifice on day 8 post-challenge.

5.3.6 rPPRV-C77c Nigeria 75/1 vaccination has precluded serological differentiation from the conventional vaccinated and challenged goats

The PPRV H protein-specific antibody response in serum collected from vaccinated and challenged animals was assessed using the commercially available c-H ELISA kit (BDSL, UK). All animals were sero-negative for PPRV specific antibodies on the day of vaccination (Figure 5.7). Unexpectedly, both the recombinant and conventional vaccinated animals were positive for PPRV H protein-specific antibodies starting from 8 dpv and the antibody levels reached a peak by 2 weeks post-vaccination, after which the level was maintained. Two unvaccinated control goats (G 2 and G 3) became seropositive by 8 dpc with the remaining two goats being border-line for serological positivity on the day they were terminated with severe clinical disease. Significant differences in the PI values were seen (2way ANOVA at $p<0.0001$) among the vaccinated (rPPRV-C77c Nigeria 75/1 and PPRV Nigeria 75/1) and control groups from 8 dpv throughout all of the sampling points. However, no significant difference ($P > 0.05$) in the proportion of PI values was observed between the two vaccinated groups for any sampling day during the vaccination and challenge periods.

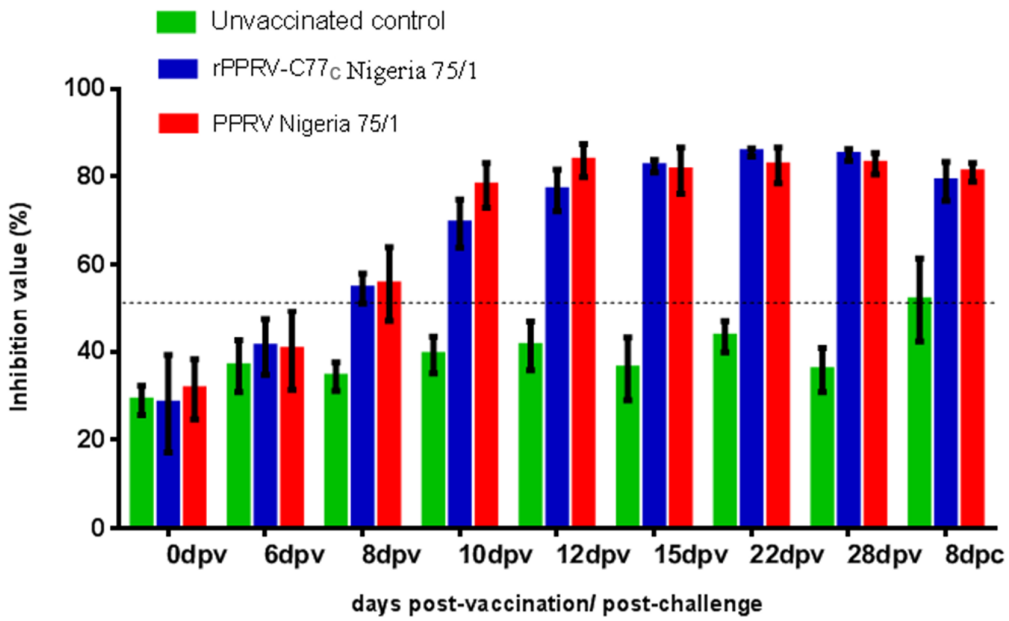

Figure 5.7 Detection of PPRV H specific antibodies in vaccinated and challenged goats. Mean percent inhibition values of PPRV H-specific antibody responses in animals vaccinated with the wild type vaccine, rPPRV-C77c Nigeria 75/1 and unvaccinated animals as determined by c-H ELISA. Serum was collected on days 0, 6, 8, 10, 12, 15, 22 and 28 days post vaccination as well as on day 8 post-challenge. The 50% inhibition value is considered as the cut-off titre and is indicated by a dotted line. The mean percent inhibition values with ± standard errors of mean are presented.

5.4 Discussion

Recent studies reporting the rescue of PPRV expressing GFP or FMDV VP1 proteins have been limited to *in vitro* data in their assessment of the protective efficacy of vaccines against challenge (Hu *et al.*, 2012b; Yin *et al.*, 2014). In the present study *in vivo* data has been generated to demonstrate that the rPPRV-C77c Nigeria 75/1 virus is able to generate adequate protection from virulent challenge in the natural host for PPRV, small ruminants. *In vivo* assessment is a necessary prerequisite to determine the utility of recombinant versions of vaccines prior to further development and licencing in the field. Both the parental (PPRV Nigeria 75/1) and mutated (rPPRV-C77c Nigeria 75/1) vaccines provided complete protection against a lethal dose of PPRV challenge. None of the animals in either vaccinated group showed any evidence of PPR disease and survived the challenge with pathogenic PPRV without any systemic replication of the virus. Furthermore, there was no transmission of vaccine virus to in-contact unvaccinated control animals housed with the vaccinated animals during the 4 week period post-vaccination demonstrating that both the parent and mutated (rPPRV-C77c Nigeria 75/1) vaccines are highly unlikely to transmit between animals and potentially revert to virulence in the field. Indeed, none of the control animals seroconverted and all developed clinical disease following challenge. High titre PPRV specific serological responses were demonstrated at 28 dpv by both VNT and ELISA for all vaccinated animals and the antibody levels in vaccinated goats was not increased even after challenge. This maintained antibody level without any virus replication in the vaccinated animals after challenge suggests the provision of sterile immunity by both of the vaccines. Further analysis of whole blood samples in FACS staining and total leucocyte counts enabled the demonstration of leukopenia in the control animals

from 4 dpc and suggested that this response may be linked to the reduction in $CD4^+$ cells as determined FACS analysis of samples from the post-challenge period. However the huge loss of leucocytes in the non-vaccinated, challenged control animals might be due to the death of granulocytes (neutrophils) alongside the death of the $CD4^+$ cells. Total leucocyte counts demonstrated a transient leukopenia in both the vaccinated groups similar to Rajak *et al* (Rajak *et al.*, 2005a).

Although the rPPRV-C77c Nigeria 75/1 vaccine virus was indistinguishable from the parent vaccine strain in its ability to protect animals, DIVA potential was not fulfilled with the mutations applied to the residues within the H protein using the current c-H ELISA. Previous studies with other viral vaccines such as RPV (Buczkowski *et al.*, 2012), NDV (Peeters *et al.*, 2001) and classical swine fever virus (Wehrle *et al.*, 2007) vaccines have postulated that epitope deletion may represent an efficient mechanism to generate DIVA vaccines. However, in the current study although the epitope mutation prevented binding of the C77 mAb *in vitro* it was not sufficient to enable DIVA *in vivo*. As mapped by phage display screening techniques, 6 critical residues were implicated for C77 binding. Mutation of 3 critical residues from wild type to alanine (R547, S549 and S550) prevented the binding of mAb to the H protein of the virus infected cells in immunofluorescence studies. These mutations were seen to be stable after several passages of rescued virus in cell culture. As it was not possible to rescue the virus after mutating the additional 3 critical residues (Y540, I542 and Y543), the animal experiment was carried out by vaccinating goats with the rPPRV-C77c virus containing just 3 mutated residues (R547A, S549A and S550A) (Muniraju *et al.*, 2015). It is likely that further mutations to the PPRV C77 mAb binding region are required to enable a serological

DIVA response to be generated following vaccination with a vaccine containing mutated H protein. Investigations to further explore mutation to the C77 binding region are planned. Another approach is currently underway to further develop a PPRV DIVA test using synthetic peptides specific to the C77 epitope region. The linear epitope present in the peptide may prevent the binding of the antibodies present in the serum.

Additional approaches to generate a DIVA vaccine could focus on the generation of a recombinant vaccine strain containing a chimeric N protein gene whereby the variable C-terminus of N could be swapped with that of RP. In this, the RP specific variable part of N gene could be detected by a specific ELISA (Parida *et al.*, 2007) whilst an ELISA specific for the PPRV C-terminus of the N protein (unpublished) would detect serological responses to naturally infected animals. If successful, the development of a DIVA test would be a great boon in the less developed regions of the world where PPRV represents a major obstacle to the development and maintenance of subsistence farming.

Chapter 6 Segmentation of PPRV to generate multivalent vaccines

6.1 Introduction

Several of the viral diseases that share common hosts (sheep and goat) with PPRV, including BTV, CPV, RVFV and pestiviruses, are under-reported (Kul *et al.*, 2008; Mondal *et al.*, 2009; Saravanan *et al.*, 2007). In many cases, these diseases are endemic in areas where PPRV is also endemic and as such their epidemiological distribution overlap. Importantly, current vaccination strategies are lacking for several of these diseases and where vaccines exist they often require multiple administrations which is not cost effective in resource-limited settings. The development and application of multivalent vaccines in endemic regions would therefore greatly reduce the cost, and give more efficient use of resources leading to the successful control of multiple diseases including those with no effective vaccines currently available. BTV, an orbivirus with a ten-segmented genome, exists in 26 serotypes and the inactivated vaccines employed in disease control programmes are expensive to produce (Calvo-Pinilla *et al.*, 2014). RVFV with a three-segmented genome (bunyavirus) is a zoonotic disease and an expensive inactivated vaccine is used to control the disease with a safe live attenuated vaccine also available (Indran, 2012). With the existence of effective live attenuated CPV vaccines, glycoproteins of PPRV have been added to CPV based recombinant thermostable bivalent vaccines, and protection against PPRV has been demonstrated (Berhe *et al.*, 2003a; Chen *et al.*, 2010; Diallo *et al.*, 2002a). However, recombinant CPV vaccines expressing the F and H proteins of PPRV require multiple administrations and do not wholly protect against virulent PPRV challenge due to the presence of pre-existing immunity to CPV (Caufour *et al.*, 2014).

Paramyxoviruses such as MV and NDV (NDV belonging to the genus *Avulavirus*) were developed as viral vectors expressing transgenes and have been proven to be safe and efficient (Brandler *et al.*, 2013; Bukreyev *et al.*, 2005; Nakaya *et al.*, 2001; Singh *et al.*, 1999). Further, a novel genome segmentation strategy (Figure 6.1) was adopted to overcome the genome length constraint for insertion of multiple and long transgenes, as demonstrated in pioneering work with MV (Takeda *et al.*, 2006) and independently with NDV (Gao *et al.*, 2008).

This chapter describes the generation of two-segmented recombinant PPRV using reverse genetics techniques. The first segment incorporated eGFP as a heterologous protein whilst the second segment incorporated the VP2 outer capsid protein of BTV (serotype 1). The rescue of a segmented version of PPRV expressing heterologous proteins and their *in vitro* characterisation is described in this chapter.

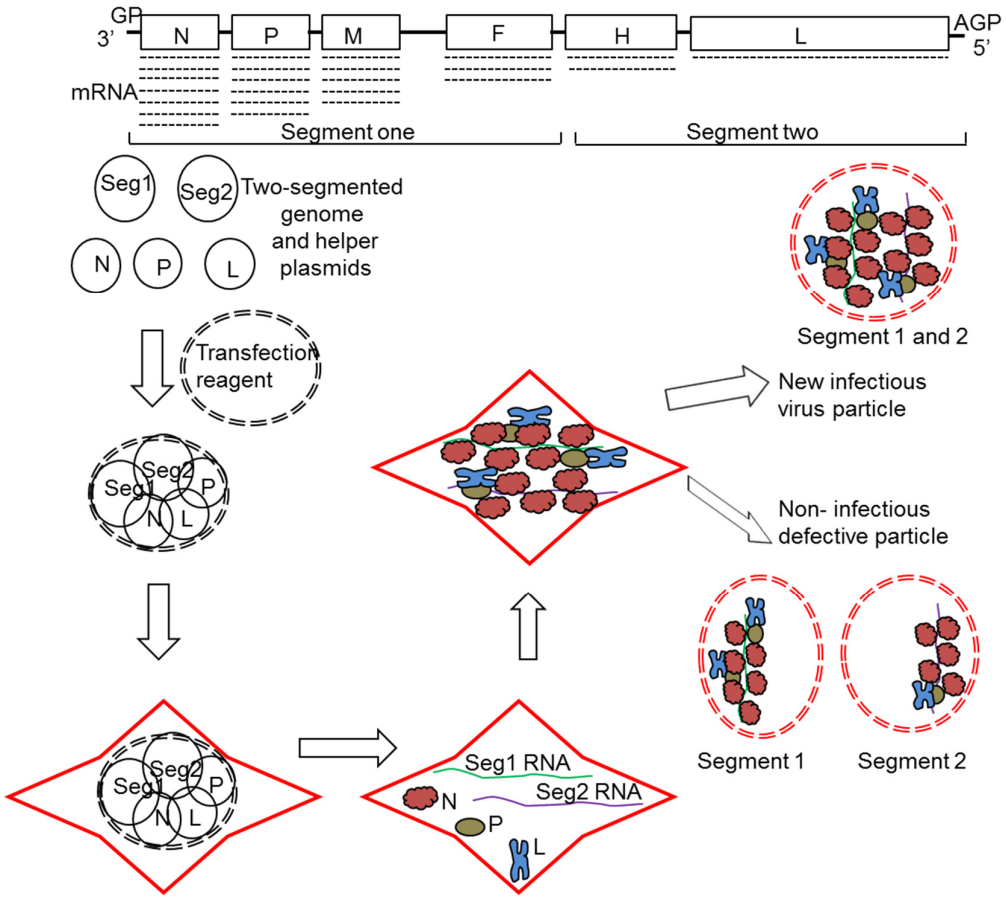

Figure 6.1 Schematic representation of a two segment strategy to generate infectious morbilliviruses with a two-segment genome. The first segment consists of the N, P, M and F genes whilst the second segment consists of the H and L genes. Recombinant viruses were rescued by transfecting both segments of the genome and the N, P and L helper plasmids into susceptible cells. The new infectious virus particle recovered contains both the genome segments. The potential for generation of non-infectious defective particles containing only a single segment exists.

6.2 Materials and methods

6.2.1 Construction of two-segmented PPRV cDNAs

Two-segment PPRV genome plasmids were constructed from the full-length cDNA plasmids of the pPPRV+GFP Nigeria 75/1 vaccine strain that already contained the eGFP gene as a heterologous transcription unit (Chapter 4). A schematic diagram showing the segmentation strategy for the PPRV genome and the steps involved in generating the segmented plasmids are shown in Figures 6.2 and 6.3. The first segment consists of the N, P, GFP, M and F genes and the second segment consists of the BTV VP2 gene alongside the H and L genes of PPRV. At the termini of each segment the authentic 3' GP and 5' AGP sequences of PPRV were retained. The presence of unique restriction enzyme sites in the PPRV full-length clone (pPPRV+GFP Nigeria 75/1) were kept intact. The segmented gene constructs were under the transcriptional control of the T7 RNA polymerase promoter in a eukaryotic expression vector backbone (pJ711) as in the parental pPPRV+GFP Nigeria 75/1. The 'rule of six' was followed in each segment to achieve efficient genome replication. Newly constructed PPRV plasmid sequences were confirmed by sequencing the modified regions on both strands.

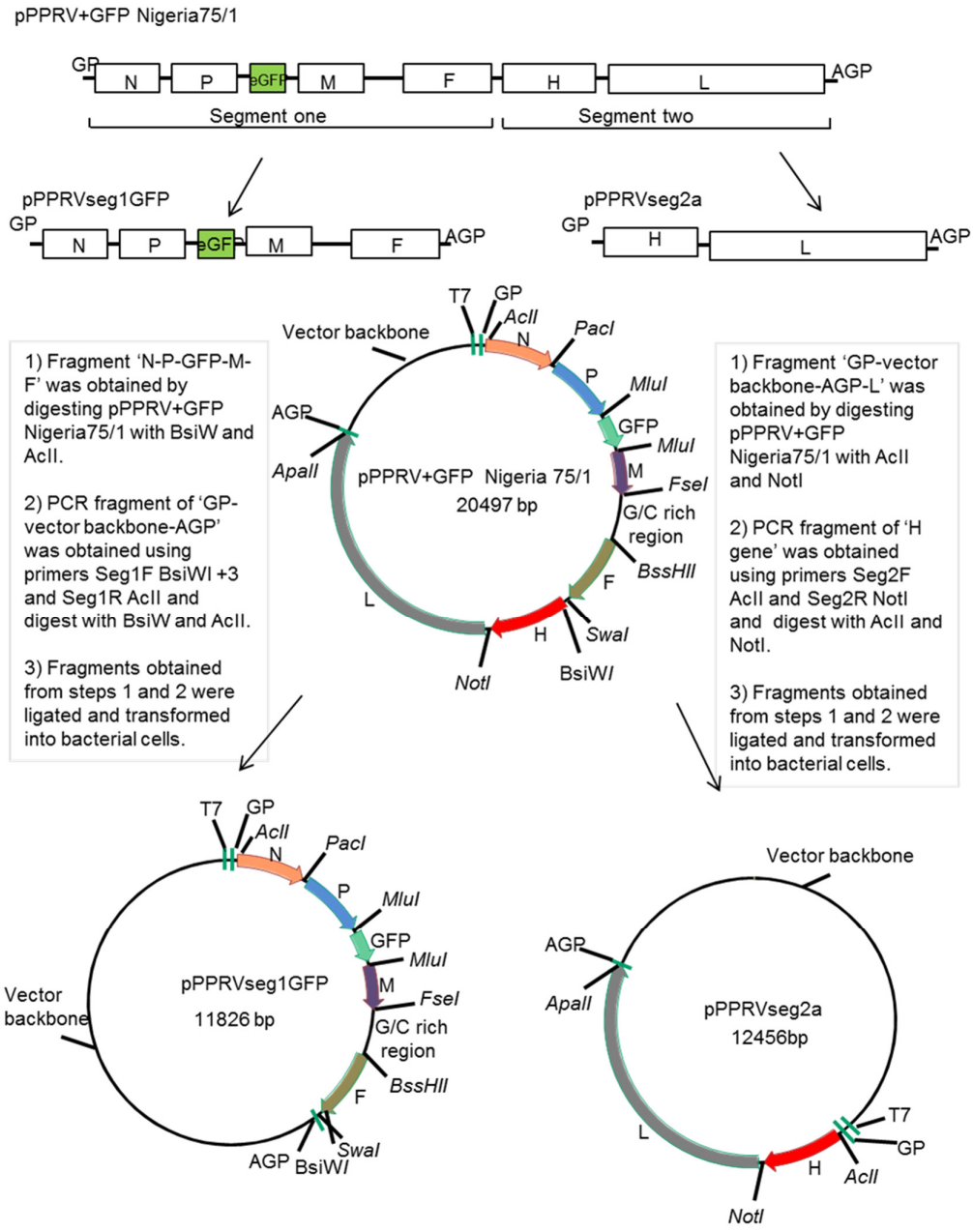

Figure 6.2 Schematic representation of the segmentation strategy and construction of a two-segmented (pPPRVseg1GFP and pPPRVseg2a) PPRV genomic plasmids.

6.2.1.1 Construction of the PPRV genome segment one plasmid, pPPRVseg1GFP

The plasmid pPPRVseg1GFP consisted of the 'GP-N-P-GFP-M-F-AGP' sequence of pPPRV+GFP Nigeria 75/1 (Figure 6.2 and Appendix VII). The following steps were followed to construct pPPRVseg1GFP.

1. pPPRV+GFP Nigeria 75/1 was digested with *Ac*II and *BsiW*I to obtain a 7,873 nucleotide long fragment containing 'N-P-GFP-M-F'.
2. Amplicons containing the 'AGP-vector backbone-GP' (3,971 nucleotides long) were amplified using primer set Seg1F *BsiW*I +3 and Seg1R *Ac*II (Appendix III) and digested with the *BsiW*I and *Ac*II enzymes. Additional nucleotides 'TGA' were inserted immediately after F gene stop codon through primer Seg1F *BsiW*I +3 to adjust the segmented genome length to make it multiple of six.
3. Fragments obtained from steps 1 and 2 were gel purified, ligated and transformed into bacterial cells, to obtain pPPRVseg1GFP. The size of the pPPRVseg1GFP plasmid was 11,826 nucleotides (Figure 6.2). The pPPRVseg1GFP was sequenced to confirm the presence of the correct nucleotide sequences and organisation of the genes.

6.2.1.2 Construction of PPRV genome segment two plasmid, pPPRVseg2a

The plasmid, pPPRVseg2a consisting of the 'GP-H-L-AGP' sequence of PPRV (Figure 6.2 and Appendix VII) was constructed following the steps as described below.

1. pPPRV+GFP Nigeria 75/1 was digested with *Ac*II and *Not*I to obtain a 10,590 nucleotide long fragment containing the 'L-AGP-vector backbone-GP'.

2. Amplicons of the 'H gene' (1873 nucleotides) were obtained using the primer set Seg2F *Ac*II and Seg2R *Not*I (Appendix III) and digested with both *Ac*II and *Not*I enzymes.

3. Fragments obtained from step 1 and 2 were gel purified, ligated and transformed into bacterial cells, to obtain pPPRVseg2a. The size of the pPPRVseg2a plasmid was 12,456 nucleotides (Figure 6.2). The plasmid, pPPRVseg2a was sequenced to confirm the presence of correct nucleotide sequences and organisation of the genes.

6.2.1.3 Insertion of the VP2 segment of BTV into pPPRVseg2a

The pPPRVseg2a construct was used to insert and express the VP2 outer capsid protein sequence of BTV (serotype 1). The VP2 CDS (2,886 nucleotide) long with 5' and 3' UTR sequences of the N gene of PPRV was inserted as a separate transcriptional unit preceding the H gene to generate pPPRVseg2bVP2 (Figure 6.3 and Appendix VII). Steps followed in the plasmid construction are described below.

1. pPPRVseg2a was digested with *Ac*II and *Not*I to obtain a 10,590 nucleotide long fragment containing the 'L-AGP-vector backbone-GP '.

2. The amplicon containing the H gene (1,925 nucleotides) was amplified using the primer set Seg2F *Pac*I and Seg2R *Not*I (Appendix III) and digested with *Pac*I and *Not*I enzymes. The H gene was removed from pPPRVseg2a and re-introduced to include the compatible UTR sequence with the preceding BTV VP2 gene. The 5' and 3' UTR sequence of the N gene were introduced between the H and the VP2 genes through PCR primers.

3. The amplicon of the ‘VP2’ CDS from BTV serotype 1 (2,886 nucleotides) was amplified using the primer set Seg2VP2F *Ac*II and Seg2VP2R *Pac*I and digested with *Ac*II and *Pac*I enzymes.

4. The three fragments obtained from the above steps 1, 2 and 3 were gel purified, ligated and transformed into bacterial cells, to generate pPPRVseg2bVP2 with the a total plasmid size of 12,456 nucleotides (Figure 6.3). The recombinant plasmid construct was sequenced to confirm the presence of the exact nucleotide sequences, gene orientation and organisation.

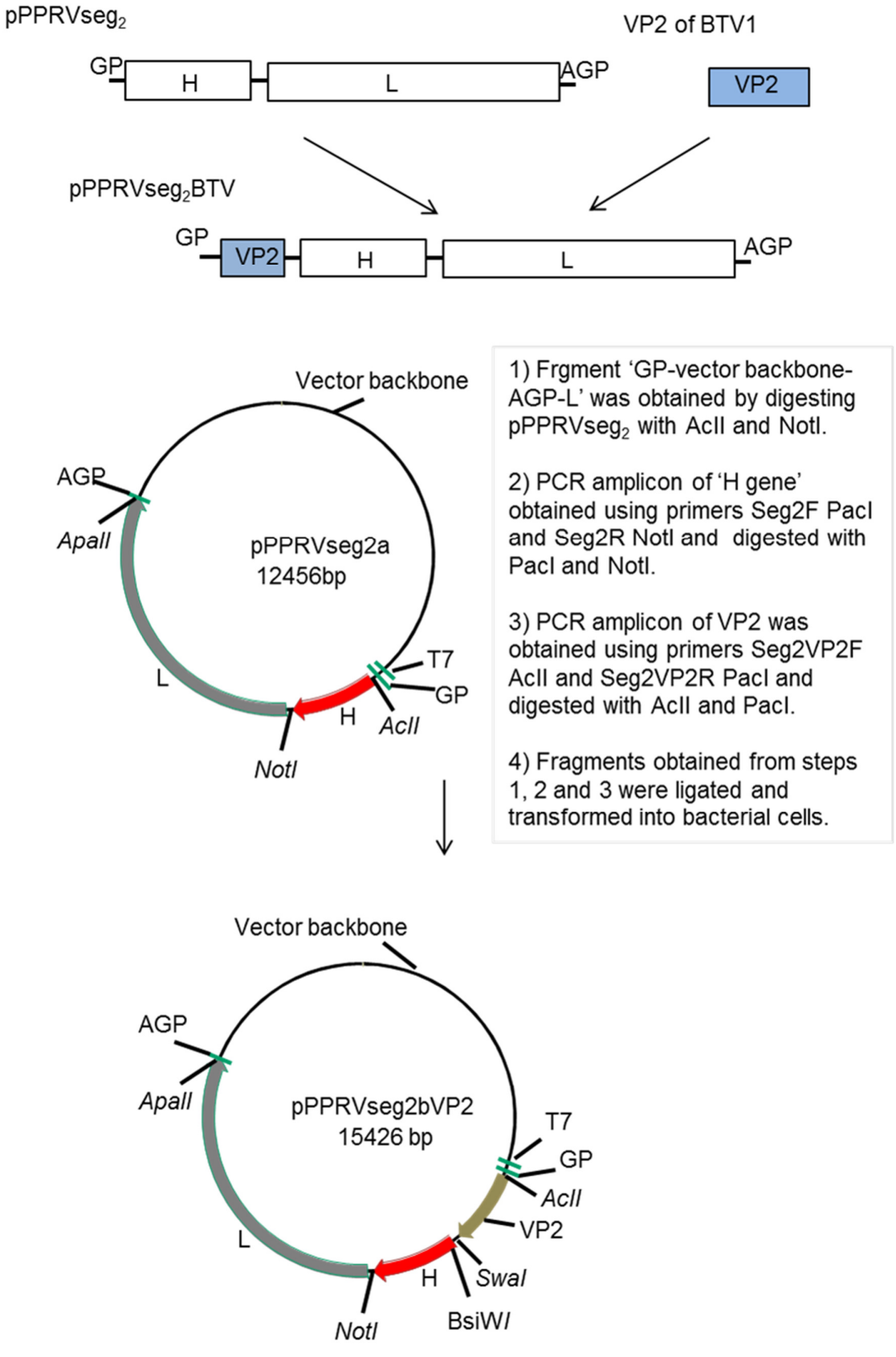

Figure 6.3 Schematic representation for the construction of a (pPPRVseg2bVP2) segmented PPRV genome with the VP2 gene of BTV.

6.2.2 Transfection and recovery of recombinant PPRVs from two-segmented genome plasmids

The two-segmented PPRV genome plasmids (pPPRVseg1GFP and pPPRVseg2a or pPPRVseg1GFP and pPPRVseg2bVP2) were transfected into VDS cells (pre-infected with rFP-T7) using TransFast transfection reagent along with the helper plasmids to generate N, P and L as described in Chapter 4 (section 4.2.4). The total amount of genomic plasmid DNA (1µg/well) needed for the transfection procedure to rescue the non-segmented PPRV, was split into two equal proportions (i.e., 0.5 µg/well) for each of the two segmented genome plasmids.

6.2.3 RT-PCR and sequencing to confirm the identity of rescued segmented viruses

RT-PCR was performed on total RNA extracted from passage 3 and passage 9 segmented viruses using following primer sets. For identification of genome segment one, forward primer PPR-F-18 (Appendix III) located on the F gene and reverse primer PPR-R-32 (Appendix III) located on the AGP were utilised to amplify the F and AGP regions. The eGFP gene from genome segment one was also amplified using primer sets PPR-F-7 and PPR-R-12 (Appendix III) located on the P and F genes, respectively. For identification of PPRV genome segment PPRVseg2a, forward primer PPR-F-1 (Appendix III) located on the GP and reverse primer PPR-R-18 (Appendix III) located on the H gene of PPRV were used to amplify GP and H regions. Amplification with primer sets PPR-F-1 and PPR-R-18 also encompassed the VP2 gene in genome segment PPRVseg2b.

6.2.4 Immunofluorescence investigations to confirm the identity of the rescued segmented viruses

To identify the segmented viruses, an immunofluorescence investigation was carried out using the anti-PPRVH mAb (C77) and an anti-BTV polyclonal antibody. Expression of GFP was observed by autofluorescence using a confocal microscope as described in chapter 4 (section 4.2.5).

6.2.5 Multi-step growth curve

Rescued segmented PPRVs and the parental un-segmented wild-type PPRV Nigeria 75/1 were grown in VDS cells to compare growth kinetics as described in chapter 2 (section 2.1.6).

6.3 Results

6.3.1 Rescue of recombinant segmented PPRVs using two-segmented genome approach

Infectious recombinant PPRV with a two-segmented genome, rPPRVsegGFP was rescued from the plasmids pPPRVseg1GFP and pPPRVseg2a using reverse genetics techniques. Further, infectious segmented PPRV containing the VP2 gene of BTV, rPPRVsegGFP+BTV was rescued from the plasmids, pPPRVseg1GFP and pPPRVseg2bVP2. The recombinant viruses showed CPE and syncytia formation from day three post-transfection in VDS cells and CPE was similar when compared to the positive control wells containing rescue of the non-segmented rPPRV Nigeria 75/1 (Figure 6.4). The rescued viruses were freeze-thawed once, clarified to remove cell debris and serially passaged in VDS cells up to passage 9.

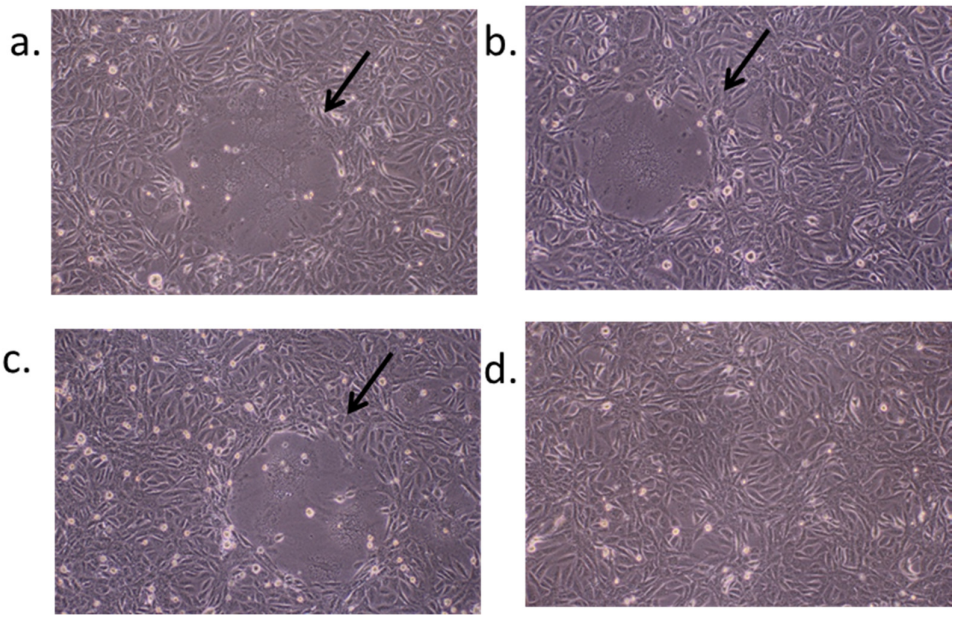

Figure 6.4 Cytopathic effect and syncytia formation of the rescued rPPRVsegGFP and rPPRVsegGFP+BTV viruses in VDS cells. Segmented or non-segmented PPRV genome plasmids were transfected into VDS cells and the rescued recombinant segmented viruses (a) rPPRVsegGFP and (b) rPPRVsegGFP+BTV showed similar syncytia formation compared with the positive control well of (c) rPPRV Nigeria 75/1. Uninfected VDS cells are shown in Figure 6.4d. Images were captured using a Nikon D7000 camera observed under light microscope (10x objective). Syncytia are indicated by arrows.

6.3.2 Confirmation of the identity of the rescued viruses

The identity of rescued viruses rPPRVsegGFP and rPPRVsegGFP+BTV at passage 3 was confirmed by RT-PCR followed by sequencing of amplified products. The PPRV genome segment specific primer sets were used in the amplification of fragments. From the genome segment one of both recombinant viruses, the 'partial F-AGP' fragment was amplified using primer sets PPR-F-18 and PPR-R-32 and also the GFP gene was amplified using the primer set PPR-F-7 and PPR-R-12. Simultaneously, using common primer sets, PPR-F-1 and PPR-R-18, the smaller 'GP-H fragment' was amplified from the rPPRVsegGFP and the larger 'GP-VP2-H fragment' was amplified from rPPRVsegGFP+BTV (Figure 6.5 and Figure 6.6). A single amplified fragment from each primer set was clearly identified with an expected fragment size as observed by agarose gel electrophoresis. The amplicons were sequenced on both the strands and no mutations were observed (Figure 6.7 and 6.8). The expected nucleotide sequences in the rescued viruses were confirmed. Similarly, the presence of the eGFP gene in segment one of both recombinant viruses was also determined by RT-PCR and sequence analysis (data not shown).

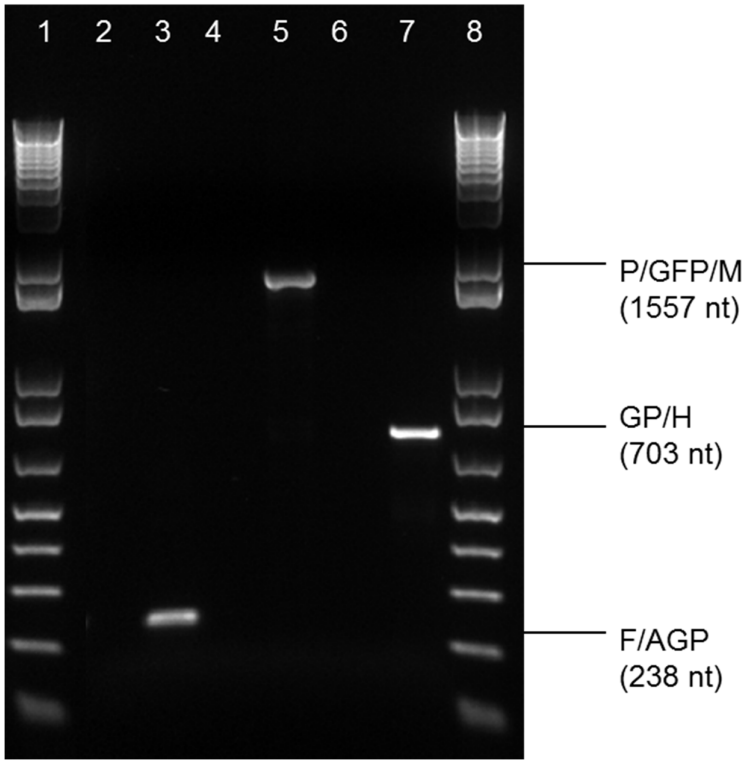

Figure 6.5 Confirmation of the identity of rescued rPPRVsegGFP virus by RT-PCR. RNA extracted from passage 3 rPPRVsegGFP was subjected to RT-PCR using genome segment specific primers. Parallel PCR without reverse transcriptase enzyme was performed to confirm that the fragments were not amplified from carry-over of genomic plasmid DNAs. Lanes 1 and 8 show the 1Kb plus marker (Appendix II). Lane 2 is a minus RT enzyme control for amplicons in lane 3 that shows the amplification of 'partial F and AGP' fragment from genome segment one using primer sets PPR-F-18 and PPR-R-32. Lane 4 is a minus RT control for the lane 5 amplification of the 'partial P, GFP and partial M' fragment from genome segment one using primer sets PPR-F-7 and PPR-R-12. Finally lane 6 is a minus RT control for the lane 7 amplification of the 'GP and partial H' fragment from genome segment two using primer sets PPR-F-1 and PPR-R-18. All primers are listed in the Appendix III. The sizes of the expected fragments are shown in parenthesis.

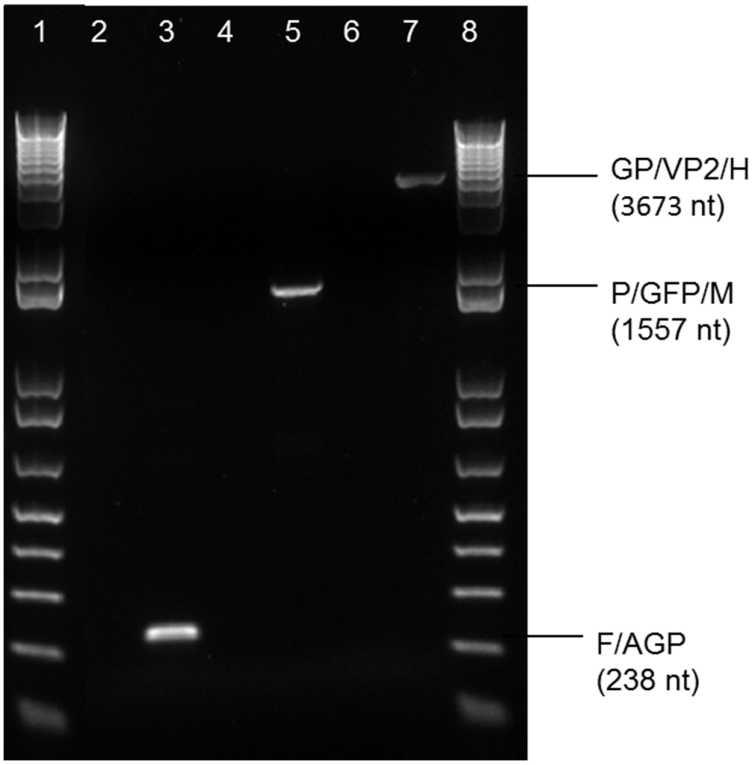

Figure 6.6 Confirmation of the identity of rescued rPPRVsegGFP+BTV virus by RT-PCR. RNA extracted from passage 3 rPPRVsegGFP+BTV was subjected to RT-PCR using genome segment specific primers. Amplifications are as detailed in Figure 6.5 with primers specific for the 'partial F-AGP' (Lane 3); 'partial P, GFP and partial M' (lane 5); and 'GP, VP2 and partial H' (lane 7). Lanes 1 and 8 represent the 1Kb marker (Appendix II). Lanes 2, 4 and 6 are RT minus controls for each amplification.

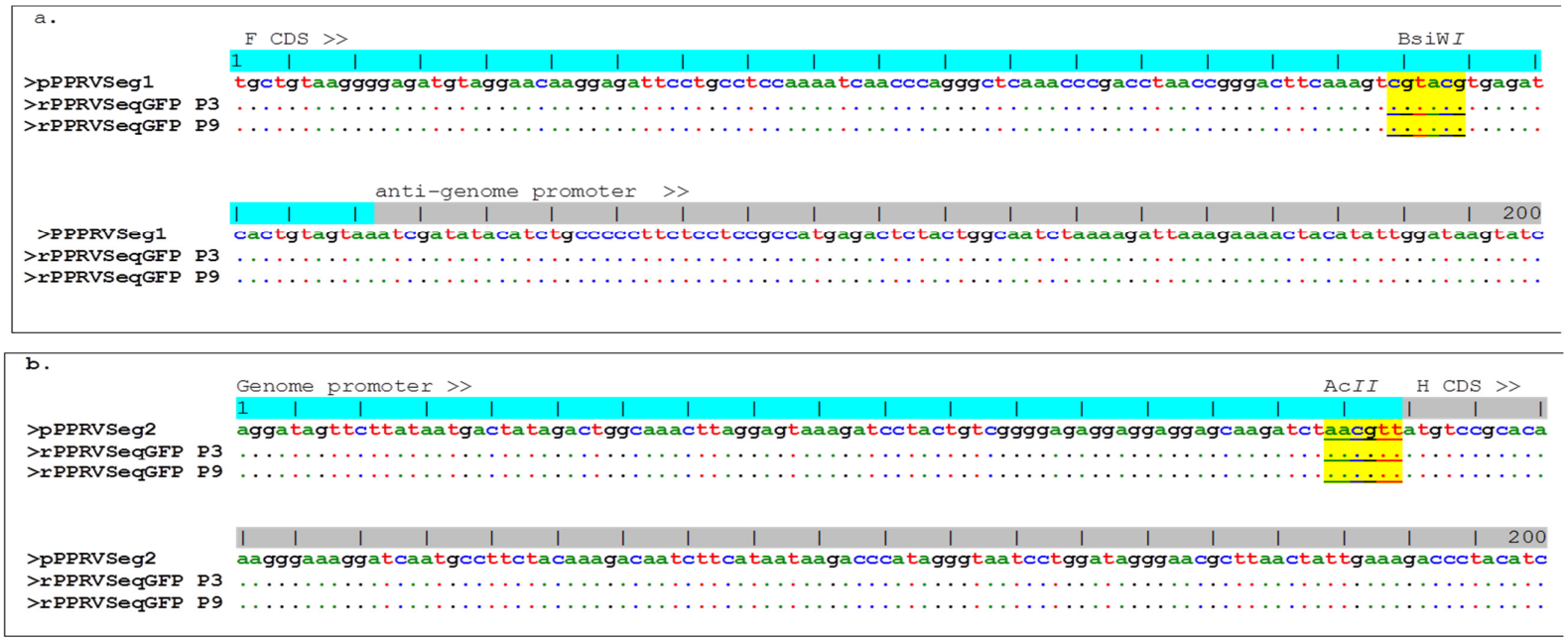

Figure 6.7 Nucleotide sequence alignments to confirm the identity of the rescued rPPRVsegGFP virus. The RT-PCR product obtained using PPRV genome segment specific primers with passage 3 and 9 of rPPRVSegGFP were sequenced and aligned with the plasmid sequence used in the transfection experiments. The alignment in panel (a) represents the PPRV genome segment one 'partial F -AGP' sequence in the plasmid pPPRVseg1GFP and those found in passage 3 and 9 of rPPRVsegGFP. Similarly, the alignment in panel (b) represents the PPRV genome segment two 'GP- partial H' sequence in the plasmid pPPRVs175eg2a and those found in passage three and nine of rPPRVsegGFP.

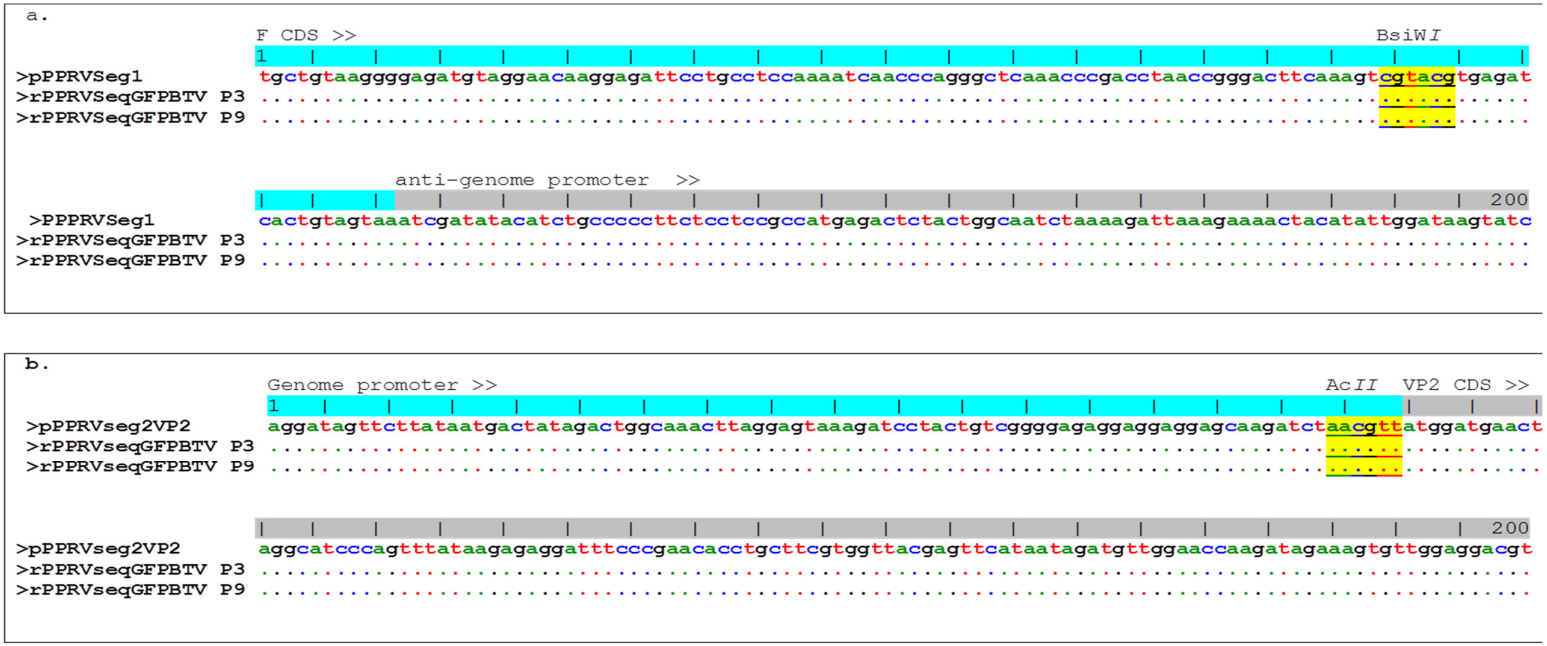

Figure 6.8 Nucleotide sequence alignments to confirm the identity of the rescued rPPRVsegGFP+BTV virus. The RT-PCR product obtained using the PPRV genome segment specific primers with passage 3 and 9 of rPPRVSegGFP+BTV were sequenced and aligned with the plasmid sequences used in the transfection and rescue of recombinant virus. The alignment in panel (a) represents the PPRV genome segment one 'partial F -AGP' sequence in the plasmid pPPRVseg1GFP and those found in passage 3 and 9 of rPPRVsegGFP+BTV. Similarly, the alignment in panel (b) represents the PPRV genome segment two 'GP- partial VP2' sequence in the plasmid pPPRVseg2aVP2 and those found in passage 3 and 9 of rPPRVsegGFP+BTV.

6.3.3 Immunofluorescence investigation into the expression of both homologous and heterologous protein expression from segmented PPR viruses

Immunofluorescence imaging demonstrated the expression of the H protein of PPRV and GFP in the VDS cells infected with the rPPRVsegGFP and rPPRVsegGFP+BTV viruses (Figure 6.9). The successful expression of eGFP from an additional transcriptional unit inserted into the genome segment one of rPPRVsegGFP and rPPRVsegGFP+BTV viruses was observed through its autofluorescence. Polyclonal serum raised against BTV (whole virus) was used in Western blotting and confocal microscopic studies but was unable to detect VP2 protein expression either in the recombinant segmented PPRV or wild-type serotype 1 of BTV due to nonspecific background staining in the negative control uninfected cells (data not shown).

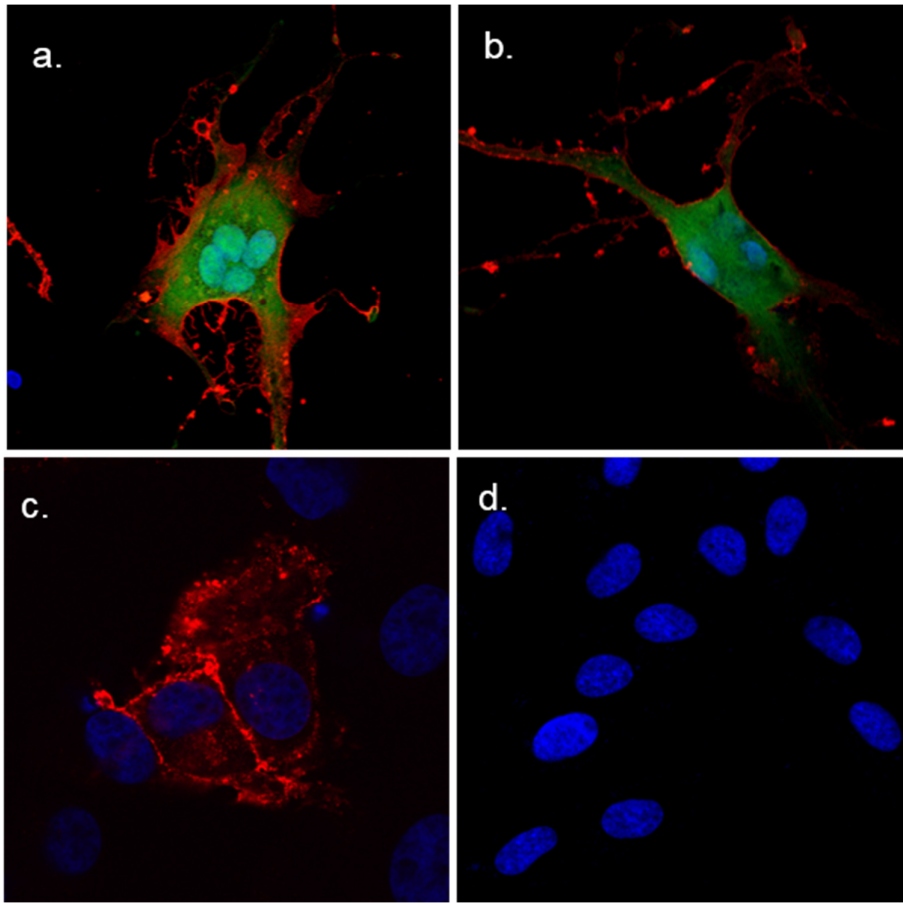

Figure 6.9 Expression of the PPRV H protein and GFP in the recombinant PPR viruses with a two-segmented genome. VDS cells were infected with (a) rPPRVsegGFP, (b) rPPRVsegGFP+BTV and (c) rPPRV Nigeria 75/1 at an MOI of 0.01 and fixed at 24 hours post-infection using 4% PFA. The uninfected cells (d) were used as a negative control. Cells were stained with mouse anti PPRV H (C77) primary mAb followed by Alexa Fluor 568 secondary antibody. Cell nuclei were stained with DAPI. Autofluorescence of GFP was visualised. Confocal laser scanning overlay of three different wavelengths – blue (405 nm) for the cell nucleus, red (568 nm) for the H protein of PPRV and green (488 nm) for GFP are shown. The H proteins of rPPRVsegGFP, rPPRVsegGFP+BTV and rPPRV Nigeria 75/1 viruses were detected using the C77 mAb. The autofluorescence of GFP was detected only for the rPPRVsegGFP and rPPRVsegGFP+BTV viruses.

6.3.4 *In vitro* growth characteristics of recombinant viruses

The replication efficiency of the recombinant PPR viruses with two-segmented genomes, rPPRVsegGFP and rPPRVsegGFP+BTV, were assessed by multistep growth curve in VDS cells and compared with the non-segmented wild-type PPRV Nigeria 75/1 vaccine strain. The rPPRVsegGFP virus exhibited a similar growth rate compared to PPRV Nigeria 75/1 with peak titres achieved at 84 hpi (Figure 6.10). The rPPRVsegGFP+BTV virus showed similar initial growth rate although the peak titre attained was approximately one $\log_{10}$ less than that of the parent rPPRVsegGFP.

6.3.5 Stability of the genome segments and maintenance of transgenes

RNAs isolated from passage nine viruses (rPPRVsegGFP and rPPRVsegGFP+BTV) were subjected to RT-PCR followed by sequencing. The PPRV genome segment specific primer sets were used in the amplification of fragments as described earlier for the passage three recombinant virus identification (Section 6.3.2). A single amplified fragment from each primer set was clearly identified with an expected fragment size (data not shown). The sequences obtained from segment one and two amplicons were aligned with passage three virus sequences and plasmid sequences as shown in Figure 6.7 and 6.8. The RT-PCR and sequence analysis confirmed the presence of GFP in rPPRVsegGFP virus and GFP and VP2 of BTV in rPPRVsegGFP+BTV virus after nine passages.

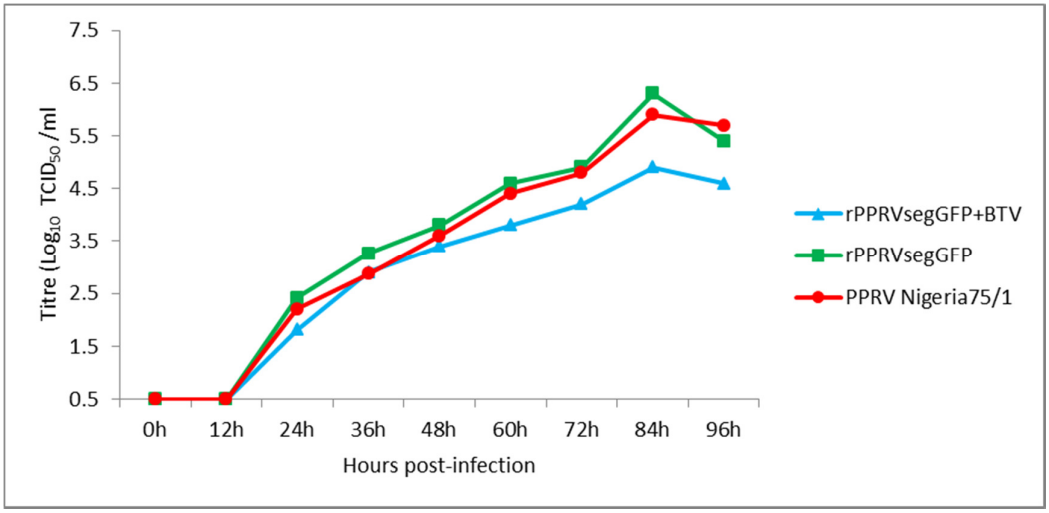

Figure 6.10 Growth kinetics of the segmented and non-segmented PPRVs in cell culture. A multi-step growth curve was carried out by infecting VDS cells with segmented rPPRVsegGFP and rPPRVsegGFP+BTV viruses and non-segmented wild-type PPRV Nigeria 75/1 at an MOI of 0.01. Viruses were grown for different time periods and samples taken at timepoints were titrated to determine viral titre at each timepoint ($TCID_{50}$).

6.4 Discussion

Establishment of a reverse genetics system for PPRV facilitates the manipulation of the virus genome to make it either positively or negatively marked (as discussed in Chapter 4). Further, this study has investigated the use of this full-length cDNA clone as a viral vector to develop multivalent vaccines expressing foreign immunogens of other viruses of relevance to small ruminant agriculture in PPRV endemic regions. It is known from previous studies that altering the transcriptional gradient in negative strand viruses, including that for PPRV, through the insertion of multiple transgenes can lead to the further polar-attenuation of genes. Insertion of a transgene at the 3' proximal end of paramyxovirus genomes provides a high level expression at the cost of virus propagation. In contrast, whilst gene insertion at 5' end has less effect on viral fitness, it can result in inadequate expression of the heterologous gene (Walsh *et al.*, 2000a; Zhao and Peeters, 2003). In addition, where expressing extra genes from a non-segmented genome, the overall genome length, following extension, can have a detrimental impact on ability of a virus to function, often through the increased likelihood of internal deletion (Rima *et al.*, 1997). Therefore, a segmented genome approach was chosen for this study PPRV to develop a multivalent vaccine.

As both PPRV and BTV circulate with a similar epidemiological distribution and a bivalent vaccine for both viruses would be of great use, a segmented PPRV expressing a BTV outer capsid protein (VP2) was generated and characterised *in vitro*. Following from the published MV and NDV genome segmentation approaches, a two-segmented PPRV rescued was attempted (pPPRVseg1GFP and pPPRVseg2bVP2) incorporating both eGFP and the VP2 protein of BTV. The

heterologous genes were expressed from different plasmids with pPPRVseg1GFP consisting of 'GP-N-P-GFP-M-F-AGP' and pPPRVseg2bVP2 consisting of 'GP-VP2-H-L-AGP'. In this way the genes, and subsequently translated proteins, required for viable virus production were present across the two segments meaning that both segments were required for true functionality. For MV whilst a two-segmented version was efficiently rescued, a three-segment strategy (N-P, M-F and H-L) led to the production of unwanted satellite bodies (Takeda *et al.*, 2006). For NDV, a two-segment strategy (N-P-L and P-M-F) that contrasts slightly with the approach taken in the present study led to a loss of virus yield following extensive passage (Gao *et al.*, 2008). As indicated, in this study the essential viral proteins (N, P and L) required for the formation of the RNP complex and efficient viral growth are located on two different segments. Though each RNA genome segment will be replicated and transcribed independently, the proteins from each segments produced in a single infected cell should complete the virus life cycle and will produce infectious particles. However, for the production of infectious virus during limit dilution passage the virions should contain both genome segments to enable viable virus production. Where genetic material for only one segment becomes packaged the defective particles may be capable of infecting new cells following fusion due to the presence of both glycoproteins on the envelope of budded virion although the incoming genetic material will not be sufficient to establish viral replication.

The rescued segmented viruses (rPPRVsegGFP and rPPRVsegGFP+BTV) were phenotypically similar to the non-segmented wild-type PPRV Nigeria 75/1 *in vitro*. The growth characteristics of rPPRVsegGFP were similar to the non-segmented PPRV Nigeria 75/1 and to that reported for the recombinant MV and NDV

segmented viruses (Gao *et al.*, 2008; Takeda *et al.*, 2006). However, the rPPRVsegGFP+BTV with an additional VP2 transgene of around 3Kbp had a slightly reduced final yield. The conservation of both the genome segments and inserted transgenes in the recombinant segmented PPRV over multiple passages reflects its stability and effectiveness for use as a viral vector. Although the presence of VP2 of BTV was confirmed in the rescued segmented virus and the expression of GFP was confirmed by autofluorescence, the expression of VP2 of BTV has not been confirmed in this study due to the lack of of VP2-specific antibodies for BTV serotype 1. A polyclonal serum raised against BTV (whole virus) was used in Western blotting and confocal microscopy but did not detect the expression of VP2 protein either in the rescued segmented PPRV or in the wild-type serotype 1 BTV infected cells. After confirming the expression of VP2 protein of BTV, the segmented PPRV needs to be assessed further for *in vivo* safety, compatibility and vaccine potency, at least for BTV as recombinant non-segmented PPRV can provided full protection in goats. This approach could be further extended to express multiple transgenes from BTV, RVFV and CPV, to generate multivalent vaccines for the control diseases of small ruminants in PPRV endemic areas.

Chapter 7 General discussion and further perspectives

PPR is a highly contagious disease, widely distributed in Africa, the Middle East and Asia where the small scale and marginal farmers are mainly dependent on goat and sheep rearing for their livelihoods. Outbreaks of PPRV can cause 100% mortality and morbidity, and as such can severely affect small ruminant production and sustainability.

PPRV has caused several serious epidemics over the last 3 decades and since 1993, sub-Saharan Africa, the Middle East and major parts of the Indian subcontinent have been considered as endemic, reporting frequent disease outbreaks and huge economic losses (Dhar *et al.*, 2002). The detection of PPRV or antibodies against PPRV has been reported from almost all African countries with the exception of vast territories across Southern Africa (Libeau *et al.*, 2014). It is unclear what factors have favoured the emergence and spread of the disease, but millions of small ruminants in southern Africa, central Asia, Southeast Asia, China, the European part of Turkey and Southern Europe must now be considered at high risk from PPRV incursion (FAO, 2013). The molecular epidemiology of PPRV based on the partial F and N gene sequence has been routinely used for the detection of virus lineage. However, these studies are not enough for understanding PPRV evolution and spread. With the development of advanced sequencing technologies, molecular epidemiological studies of viruses have started to utilise whole gene, and even complete genome data. Historically, full sequence data was only available for three lineages of PPRV (lineage-I, II and IV). In this study, full genome data was generated for all four lineages and was utilised to investigate the evolutionary dynamics of PPRV through time and space (Muniraju *et al.*, 2014). The increased

data has enabled a more precise evolutionary and phylogenetic assessment of the relationships between lineages by reducing the associated estimation errors resulting in increased confidence in estimates. A Bayesian phylogenetic analysis of all PPRV lineages mapped the time to most recent common ancestor and initial divergence of PPRV to a lineage III isolate at the beginning of 20th century. Substitution rates are critical parameters for understanding virus evolution because restrictions in genetic variation can lead to lower adaptability and pathogenicity. The nucleotide substitution rate throughout the complete genome for PPRV was found to be similar to that predicted for other paramyxoviruses (10^{-3} to 10^{-4} substitution/site/year) (Furuse *et al.*, 2010; Jenkins *et al.*, 2002; Pomeroy *et al.*, 2008; Wertheim and Pond, 2011). The demographic analysis of PPRV indicated an increased divergence during the post-rinderpest eradication era, however the recent decline observed in viral genetic diversity could be due to active efforts in the control and eradication of PPRV. A phylogeographic approach estimated the probability for the root location of an ancestral PPRV and individual lineages as being Nigeria for PPRV, Senegal for lineage I, Nigeria/Ghana for lineage II, Sudan for lineage III, and India for lineage IV. This suggests that the origin of PPRV was in western Africa with subsequent spread to eastern Africa, the Middle East, and Asia. However, the PPRV genome sequence data representing the entire geographic region needs to be enhanced further to provide greater confidence in such phylogenetic assessments.

From the perspective of all lineages across endemic areas PPRV has been controlled using two existing commercially available live attenuated PPRV (Nigeria 75/1, Sungri 96) vaccine strains. Although these are safe and efficacious vaccines, due to generally inconsistent vaccination strategies in endemic countries PPR has continued

to emerge. No tools currently exist that allow serological differentiation between vaccinated and naturally infected animals. Marker vaccines are a potential solution to the DIVA concept that may help in PPR control programmes, especially if eradication efforts increase. Marker vaccines along with companion DIVA tests are especially required for sero-surveillance during the final stage of any eradication programme to enable declarations of freedom from disease to be made. Without a marker vaccine and DIVA test, the time taken and costs incurred in ensuring serological freedom from natural infection following the cessation of vaccination are increased significantly. This was exemplified by the rinderpest eradication programme where serological surveillance in the absence of vaccination was costly and took many years. The current commercially available PPRV diagnostic ELISAs target the N and H proteins and detect antibodies in vaccinated as well as naturally infected animals. However more recently, some DIVA strategies for PPRV have focussed on developing protein subunit vaccines expressing the PPRV F and or H gene in viral vectors like pox viruses (vaccinia, Capri pox and fowl pox) and adenoviruses (canine or human type) (Chandran *et al.*, 2010; Chen *et al.*, 2010; Diallo *et al.*, 2002a; Herbert *et al.*, 2014; Rojas *et al.*, 2014). Here the absence of the PPRV N protein in the vaccine preparation facilitates positive serological identification of naturally infected animals. However, such subunit vaccines often require multiple doses and may have reduced efficacy due to: (i) potential pre-existing immunity to the viral vector (Caufour *et al.*, 2014); (ii) the potential for short lived antibody responses; (iii) potentially high costs of recombinant vaccine production. An alternative strategy is to use the existing live attenuated PPRV vaccine and manipulate a specific region or epitope of a viral protein to obtain positively and/or negatively marked vaccines.

Using reverse genetics techniques RNA virus genomes can be modified through cDNA copies of their genome. Using this technique, a RPV vector based chimeric marker vaccine was developed for PPRV by replacing the F and H or F, H and M genes of RPV with those from PPRV. Both chimeric vaccines elicited protective immune responses in goats challenged with virulent PPRV (Das *et al.*, 2000; Mahapatra *et al.*, 2006). Despite *in vitro* and *in vivo* characterisation, the abrogation of vaccination during the final stages of the RPV eradication campaign precluded the use of these chimeric vaccines. However, the establishment of reverse genetics for PPRV had been unsuccessful until recently (Hu *et al.*, 2012a; Muniraju *et al.*, 2012). Previously, only minigenome systems and the transient expression of different viral proteins had been reported (Bailey *et al.*, 2007). Previous attempts to generate a reverse genetics system for PPRV had failed, most likely due to errors in the sequence of the cDNA clone (Bailey, 2006). Certainly, the availability of a correct complete genome sequence is critical for successful establishment of PPRV reverse genetics system and recent technological advances have enabled the generation of full genome sequence with relative ease, as described in chapter 4. Alongside this the advances in DNA synthesis have enabled the synthesis of full length genomes in a more efficient manner. Further, commercial genome synthesis has avoided the introduction of unintentional mutations during cloning and ligation procedures that may affect the rescue of virus.

As a proof of principle, the full genome cDNA of PPRV of one of the most widely used vaccine strains (Nigeria 75/1) was synthesised and rescued using helper plasmids (Muniraju *et al.*, 2015). Three recombinant viruses (rPPRV Nigeria 75/1, rPPRV+GFP Nigeria 75/1 and rPPRV-C77c Nigeria 75/1) were recovered from the

cDNA clones. Of these rPPRV+GFP Nigeria 75/1 has attributes that may enable its use as a positively marked vaccine. The recombinant PPRV Nigeria 75/1 vaccine strain expressing GFP rescued by Hu *et al* (Hu *et al.*, 2012a) was reported as an aid to the detection of CPE through the expression of GFP in infected cells to optimise the OIE accredited VNT. The expression of a GFP maker will enable greater confidence in the evidence for virus infection without the need to test for neutralising antibodies in sera. Further, the creation of GFP tagged virulent PPRV was proposed as a research tool for the evaluation of host-pathogen interactions as described previously for measles virus (de Vries *et al.*, 2010).

It was hypothesised that the approach taken with rPPRV-C77c Nigeria 75/1 would enable the generation of a negatively marked vaccine that would allow differentiation between vaccinated and naturally infected animals through the response to the mutated H protein. As the C77 mAb binding site had been mutated, it was anticipated that only sera from naturally infected animals would compete with the C77 mAb to bind whole virus antigen whilst sera from animals vaccinated with the recombinant virus containing the mutated H protein would be less able to compete and bind. However, whilst this hypothesis worked in an *in vitro* immunofluorescence study, the *in vivo* study demonstrated that although the vaccinated animals were fully protected against virulent virus challenge, the antibodies raised against rPPRV- C77c Nigeria 75/1 virus in goats were able to bind to the antigen used in the c-H ELISA indicating incomplete abrogation of the binding site of the C77 antibody. Alternatively it is possible that the polyclonal antibodies elicited against alternative, potentially overlapping epitopes present in this region of the mature protein bound to the H protein present in the whole virus

antigen preparation used in the ELISA. This could still prevent the binding of the C77 mAb through steric hindrance. As only three residues were mutated to generate rPPRV- C77c Nigeria 75/1 it is plausible that further mutations in this area may enable the fulfilment of DIVA requisites with this approach.

Should this strategy fail, other approaches may be possible to enable generation of a DIVA vaccine. Such strategies include generation of a virus containing a chimeric N gene such that the hyper-variable region at the C-terminus of the N protein is not recognised in an ELISA developed against the PPRV C-terminus of the N protein. By exchanging this region with that of RPV this could enable the use of an existing rinderpest recombinant N protein-based indirect ELISA (Parida *et al.*, 2007) to detect PPRV vaccinated animals whilst the PPR recombinant N protein ELISA (unpublished data) would detect only naturally infected animals. Although PPRV and RPV are antigenically closely related, the two ELISAs based on the highly variable region of the C-terminal of the N proteins of PPRV and RPV have been shown to be specific and not cross-reactive. In the absence of DIVA, another approach would be to use large ruminant serosurveillance as an indicator for the circulation of wildtype virus in areas where small ruminants are regularly vaccinated. Large ruminants can be subclinically infected and develop antibodies against PPRV (Balamurugan *et al.*, 2014; Balamurugan *et al.*, 2012a; Khan *et al.*, 2008; Sen *et al.*, 2014). Sen *et al* (2014) recently reported the transmission of PPRV from clinically infected goats to cattle with the recovery of PPRV from these sub-clinically infected cattle after 3 weeks of direct contact challenge. In developing countries, both large and small ruminants are generally farmed together within close contact, often sharing enclosed habitations as well as pasture and drinking areas. Such scenarios provide

ideal opportunities for the transmission of wild type virus between these animal populations. A recent study assessing cattle and buffalo populations in an endemic area of Pakistan (unpublished data) and Tanzania (Lembo *et al.*, 2013) demonstrated serological detection of PPRV antibodies in cattle and buffalo that may help to identify PPR virus circulation in such localities in the absence of clinical disease indicators in small ruminant populations.

A further aspect of this study sought to increase the valency of the current live attenuated PPRV vaccines by generating segmented versions of PPRV expressing heterologous proteins from other small ruminant pathogens of importance in PPRV endemic areas. This was used to evaluate the potential for the utilisation of a single dose live attenuated vaccine against more than one disease to reduce the cost and improve the efficiency of vaccination programmes. Paramyxoviruses such as MV and NDV have been proven to be safe and efficient vaccine vectors and have been used to develop multivalent vaccines expressing foreign antigen from a wide range of related viruses (Brandler *et al.*, 2013; Bukreyev *et al.*, 2005; Nakaya *et al.*, 2001; Singh *et al.*, 1999). In this study, a recombinant two-segmented PPRV was created, with one segment carrying the VP2 outer capsid protein of BTV and the other GFP. The recombinant rPPRVsegGFP+BTV was successfully recovered from the cDNA clone. This virus needs to be characterised further *in vitro* for assessment of transgene expression and *in vivo* for vaccine safety and potency for PPRV and BTV. Further, in two additional recombinant viruses the GFP gene on segment one was replaced with GcGn gene of RVFV and the P32 gene of CPV (Figure 7.1).

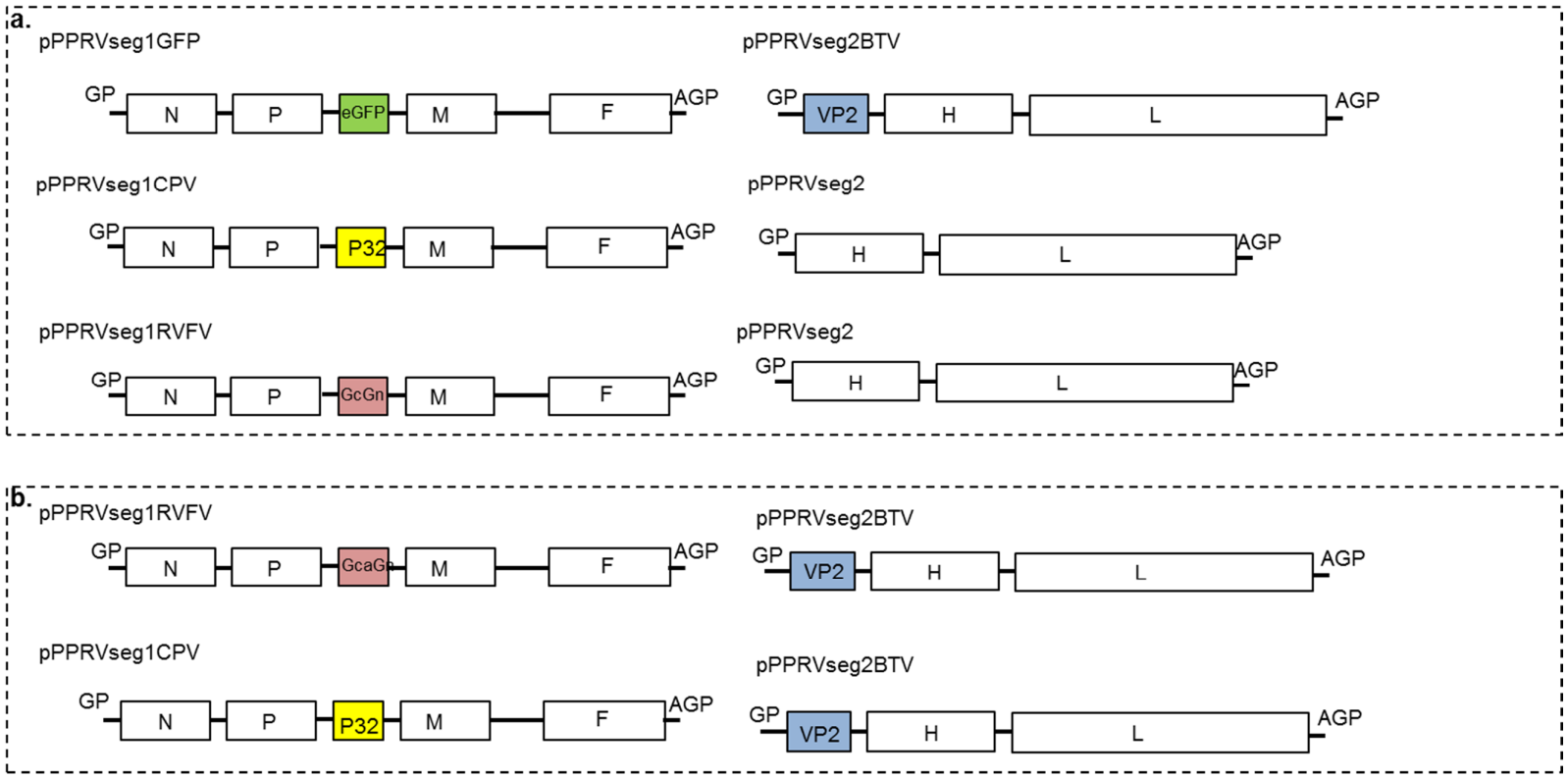

Figure 7.1 A schematic representation of approaches to develop PPRV vector based (a) bivalent or (b) trivalent vaccines expressing antigens of GcGn from RVFV, P32 from CPV and VP2 from BTV.

However transfection experiments to recover virus from the cDNA clones were not completed due to time constraints. Utilising a combination of PPRV segments harbouring either the RVFV or CPV antigen on segment one and the BTV antigen on segment two paves the way for potential bivalent or even trivalent vaccines to be developed (Figure 7.1). Alternatively, by mixing these rescued viruses a tetravalent vaccine could be produced for PPRV, BTV, CPV and RVFV and the multivalent vaccines could be used according to the circulation of viruses in a particular geographical location. Further work is planned to evaluate this area of study that could have important future ramifications on the small ruminant sector in PPRV endemic regions.

References

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Appendix

I. Solutions and reagents

TAE (Tris-acetate EDTA) buffer (50X)

242g	Tris
18.61g	EDTA
57ml	Glacial acetic acid
Make up to 1L with water	

TSS solution

12 ml	LB (2X) medium
8ml	10% PEG + 60mM $MgSO_4$ (3X)
1.2ml	DMSO
2.8ml	water

10% PEG+60mM $MgSO_4$ (3X)

90g	PEG8000
192ml	water
18ml	1M $MgSO_4$

TCM solution

0.25ml	1M $CaCl_2$
0.25ml	1M $MgCl_2$
0.25ml	1M Tris/Cl
Make up to 25ml with water	

SOC media

50µl	IM Glucose
25 µl	IM$MgCl_2$
25 µl	lMMgS0$_4$
24ml	LB (1X) medium

Growth medium

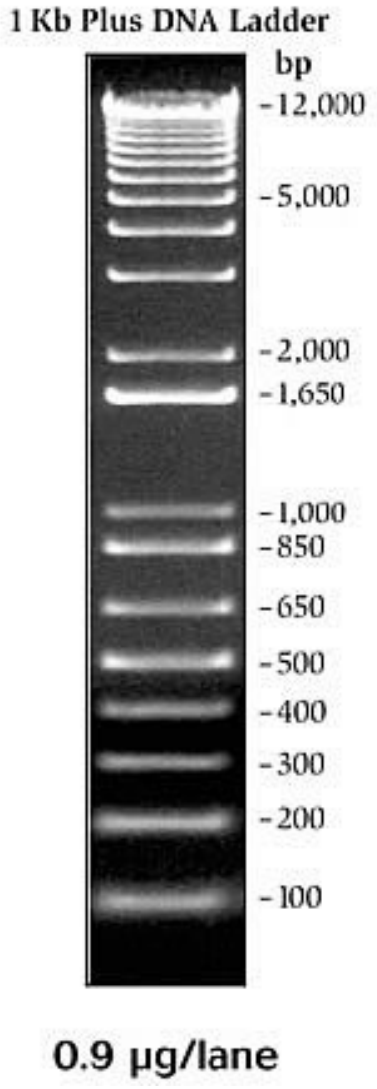
450ml	DMEM
50ml	Fetal calf serum
5ml	Penstrep (Sigma 100,000 IU/ml)

RBC lysis buffer (10x)

8.02gm	NH4Cl (ammonium chloride)
0.84gm	NaHCO3 (sodium bicarbonate)
0.37gm	EDTA (disodium)

Make up to 1L with water.

II. DNA marker 1 kb plus (Invitrogen)



III. Primer sequences

List of PPRV genome specific primers used for amplification and sequencing

Name	Sequence	Name	Sequence
PPR-F-P1	accaaacaaagttgggtaagga	PPR-R-P1	cgaacaaagataacatgctgatc
PPR-F-P2	gaagagttcaatatgtt(g/a)ttag	PPR-R-P2	agtatgagagataccatgaac
PPR-F-P3	tcctctggagctatgc(g/c)at	PPR-R-P3	gcgatttccgagactagtt
PPR-F-P4	aagattctggagg(a/g)ccagg	PPR-R-P4	ttgagagacttgatacattcc
PPR-F-P5	aactctcaagtacagcgttac	PPR-R-P5	ggagttaagaacttcttgaat
PPR-F-P6	aggagtgcaaagacgatcc	PPR-R-P6	ggagtcattctctttaggct
PPR-F-P7	tccagctaaaacctgt(c/t)ga	PPR-R-P7	ttagcatcttgtggaattcc
PPR-F-P8	agtgattgaggataacgac	PPR-R-P8	ttagcgctaaacacacttcc
PPR-F-P9	tacacgtgggcaactttag	PPR-R-P9	agatatcggttaagatcctc
PPR-F-P10	gtcatcatcaacgatga(t/c)caa	PPR-R-P10	cctaagttttgtttgatgatg
PPR-F-P11	cgcagaaaggaag(g/c)ag(a/g)ca	PPR-R-P11	ttgcgacccgtgtcatgatg
PPR-F-P12	catcatgacacgg(g/a)tc(g/c)caa	PPR-R-P12	tggtcttacattcttggttatc
PPR-F-P13	ctatatcaacaatga(g/a)cttgtc	PPR-R-P13	aatctcttgtgtccacatatgt
PPR-F-P14	tgagcccattgct(t/c)cagga	PPR-R-P14	actgccgacctgtattgtc
PPR-F-P15	gggcttgtcacattaatatgct	PPR-R-P15	atggtgattgacctttcgtc
PPR-F-P16	tccagagtcggct(g/t)aatac	PPR-R-P16	gagaaatgagctcttgttac
PPR-F-P17	gggattattcggaagaacata	PPR-R-P17	aaaatgaaggaggtcgagtc
PPR-F-P18	cattttgcaatggcac(a/g)gga	PPR-R-P18	tggaaacatcataagtggctg
PPR-F-P19	tctctgaggatagagtgttt	PPR-R-P19	ggtctggtcttcaag(a/g)acat
PPR-F-P20	tatcaaagatagctgcatatct	PPR-R-P20	aattatcacaacgaggtctc
PPR-F-P21	tcttattgatgggttcttcc	PPR-R-P21	ctgcatgccctttcatcata
PPR-F-P22	ctgtgtatagacaa(c/t)tggaa	PPR-R-P22	ttacctgacaggcccgca
PPR-F-P23	aagataaccaccgaggtg	PPR-R-P23	attagggacactgtccag(a/g)g
PPR-F-P24	gaatcgcctcactagtgca	PPR-R-P24	gatataaggatctgttggaaa
PPR-F-P25	gggtctcaattatcttaacatg	PPR-R-P25	tggtctaagatttcatgtgc
PPR-F-P26	ttgacaaagaaaggg(c/a)agtgt	PPR-R-P26	taggcccatgagtaaactg
PPR-F-P27	agtctctacatccaccaac	PPR-R-P27	tccaccaaatgtctcctgtg
PPR-F-P28	gagaaagatcacatgaatgaa	PPR-R-P28	acagagatgtttggcttgaat
PPR-F-P29	ctcacacattacatcaagg	PPR-R-P29	cagcattgaaccggaacc
PPR-F-P30	caacagcggggt(a/g)acagc	PPR-R-P30	ggcctttaatcctatcataataag
PPR-F-P31	ccattaagcaaagcataat(c/t)aga	PPR-R-P31	aagctgggaatagatacttatcc
PPR-F-P32	ggagggaatggttgttcac	PPR-R-32	accagacaaagctgggaata
PPR-F-P12b	agggacccgcccgagaacgt	PPR-R-P21B	gaatcccactcattcttcagtgca
PPR-F-P20B	caagacagagatgagatcaatga	PPR-R-P11b	catgatgtttacacatggatgtgccg
PPR-F-P11b	ccgaggccaggcgcc(g/a)ggca	PPR-R-P10b	ggaggtggtcctc(c/t)ctcggt
PPR-F-P5B	ggcacagacgggaactcagt	PPR-R-P9B	cttgggtcgggt(c/t)tgtgct
PPR-F-11C	tTgccctcgaccaccggga	PPR-R-P20B	tcgtgggtgat(a/g)aatacg
PPR-F-15B	tcaccatgtccgcacaaag	PPR-F-25B	attatgattatgaacaattc
PPR-F-P21B	acctacaattccatcactgaagga	PPR-F-29B	ctggctgcgta(c/t)aaggaagttc

List of RACE primers used to determine the 3' and 5' end of PPRV

Name	Sequence
P5-1 (5'-RACE)	caacagcaggcagcgtgaat
P5-2 (5' nest 1)	cggcactaatcagacaaacaat
P5-3 (5' nest 2)	atccaaagctgagaaactgctg
P3-1 (3'-RACE)	aacgggtaatgatggacgagtc
P3-2 (3' nested)	cgctttgtctttgttcctcttg
DT88 (adaptor)	gaagagaaggtggaaatggcgttttgg (5'-phosphorylated, 3'-end inverted dA)
DT89 (anti-adaptor)	ccaaaacgccatttccaccttctcttc

List of primers used for helper plasmids (N, P and L) cloning

Name	Sequence
N-ORF-For-Nig75	gaccatggctactctcc
N-ORF-Rev-Nig75	gtcttatcagctgaggagatcc
P-ORF-For-Nig75	accaccgatggcagaagaac
P-ORF-Rev-Nig75	ggattgttacgctgcttgg
L-ORF-SalI-For-Nig75	aggttgtagtcgacatggactcactatcagtc
L-ORF-SalI-Rev-Nig75	agatgtatgtcgacttaacctctgacgagtgc

List of primers used for C77 monoclonal antibody binding site directed mutagenesis

Name	Sequence
i) PPRV Ng75/1 H Y540A I542A Y543A	
Forward primer	gagcatgcaatcgtggcctatgccgctgacacgggt
Reverse primer	acccgtgtcagcggcataggccacgattgcatgctc
ii) PPRV Ng75/1 H R547A S549A S550A	
Forward primer	gacacgggtgcctcagcagcttacttctacccagtc
Reverse primer	gactgggtagaagtaagctgctgaggcacccgtgtc
iii) PPRV Ng75/1 H Y540A I542A Y543A R547A S549A S550A	
Forward primer	gagcatgcaatcgtggcctatgccgctgacacgggtgcctcagcagcttacttctacccagtc
Reverse primer	gagcatgcaatcgtggcctatgccgctgacacgggtgcctcagcagcttacttctacccagtc

List of primers used for PPRV genome segmentation and insertion of VP2-BTV

Name	Sequence
Seg1F BsiWI +3	gcgcgtacgtgagatcactgtagtaaatcgatatacatctgc
Seg1R AclI	Cataacgttagatcttgctcctcct
Seg2F AclI	Acgaacgttatgtccgcacaaaggga
Seg2R NotI	accatcgctcgaggcggccgccgagccat
Seg2VP2F AclI	gagcaagatctaacgttatggatgaactaggcatcc
Seg2VP2R PacI	atgtttaattaatgatttggacggagggtgcgtctgcgctcatacgttgagaagttttgt
Seg2F PacI	tcattaattaaacatcgcccgccagtattataaaaaacttaggacgaaaggtcaatcacc
Seg2R NotI	gatggctcggcggccgcctcgagcgat

IV. White blood cells count (per mm^3 blood) in goats during post vaccination and post challenge period

Period	rPPRV-C77 Nigeria 75/1				rPPRV Nigeria 75/1				Unvaccinated control			
	Goat 1	Goat 2	Goat 3	Goat 4	Goat 7	Goat 8	Goat 9	Goat 10	Goat 5	Goat 6	Goat 11	Goat 12
0dpv	13600	11800	14800	10340	15400	12040	15800	9240	14640	14900	11400	11880
2dpv	12900	11885	12000	10200	13180	11100	16260	9220	12900	14000	11550	12000
4dpv	11780	10040	11680	8160	10080	9510	10940	8160	13000	14200	13960	10080
6dpv	10560	9260	11680	7480	8080	8520	11880	7880	13740	15380	10680	10800
8dpv	12250	12960	10860	9240	12560	9620	12820	7720	14080	14420	12040	12140
10dpv	13600	12820	10520	9400	12060	10380	16500	8680	14780	16460	11860	11060
12dpv	12260	11440	11940	10180	14640	8800	16020	9780	15360	14980	12440	12420
15dpv	13040	12340	12100	10580	14540	9940	16100	9400	14140	14340	13420	12200
22dpv	14060	12760	13460	10080	14380	11000	15540	8700	13600	14540	12460	12200
28dpv/ odpc	14880	13240	11400	10420	14440	11680	17500	8200	13900	14660	11860	11980
0dpc	12880	13240	11400	10420	14440	11680	17500	8200	13900	14660	11860	11980
2dpc	10540	12380	12920	10320	14820	12280	13160	8280	13500	14620	12520	11780
4dpc	12900	11580	12180	11020	14220	11240	17000	9780	5900	6820	5600	6060
6dpc	9360	8980	8640	8100	9040	7580	12820	5780	4020	4340	3080	3380
8dpc	10780	10480	9200	8560	12340	8900	11720	7680	2640	4640	3920	3900
10dpc	10560	9900	10060	9100	11800	8400	11080	5940	NA	NA	NA	NA
12dpc	11280	10480	10260	9360	11300	8520	11860	6920	NA	NA	NA	NA

* dpv, days post vaccination; dpc, days post challenge; NA, not applicable.

V. Ct-values from reverse-transcription real-time PCR in the clinical samples of vaccine-challenge experimental animals during challenge

Treatment group	Animal no	Ct-values*																							
		0dpc			2dpc			4dpc			6dpc			8dpc			10dpc			12dpc			14dpc		
		E	N	S	E	N	S	E	N	S	E	N	S	E	N	S	E	N	S	E	N	S	E	N	S
rPPRV-C77 virus	G1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	G2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	G3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	G4	-	-	-	-	-	-	-	-	-	-	37.7	-	-	-		-	-	-	-	-	-	-	-	-
PPRV Nig75/1 vaccine	G7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32.7	-	-	-	-	-	-	-	-	-
	G8	-	-	-	-	-	-	-	-	-	-	-	-	-	38.9	-	-	39.7	-	-	-	-	-	-	-
	G9	-	-	-	-	-	-	-	-	-	-	-	33.2	-	-	-	-	-	-	-	-	-	-	-	-
	G10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control un-vaccinated	G5	-	-	-	-	-	-	37.5	-	-	29.2	30.0	25.2	24.2	21.9	19.2	NA	NA	NA	NA	NA	NA	NA	NA	NA
	G6	-	-	-	-	-	-	35.8	-	-	26.9	29.5	23.3	25.1	21.5	19.4	NA	NA	NA	NA	NA	NA	NA	NA	NA
	G11	-	-	-	-	-	-	-	40.5	-	30.4	31.8	28.1	25.5	27.0	23.5	NA	NA	NA	NA	NA	NA	NA	NA	NA
	G12	-	-	-	-	37.2	-	38.6	37.3	-	28.5	28.8	26.5	27.6	25.1	20.6	NA	NA	NA	NA	NA	NA	NA	NA	NA

*Ct-value more than 40 are not considered and represented as '-'; Eye sample (E); Nasal Sample (N); Saliva sample (S); Days post-challenge (dpc); Not applicable (NA).

VI. PPRV specific neutralising antibody titres detected in serum of individual vaccinated and unvaccinated challenged goats.

Treatment group	Animal no	Neutralisation titre (log_{10})					
		0dpv	8dpv	15dpv	28dpv	8dpc	14dpc
rPPRV-C77c Nigeria 75/1	G1	0	2.8	4.16	5.31	5.31	5.31
	G2	0	2.83	4.36	4.76	4.36	5.31
	G3	0	2.91	5.61	5.01	5.01	5.01
	G4	0	2.66	4.36	5.31	5.01	5.36
PPRV Nigeria 75/1	G7	0	2.83	4.16	5.06	5.27	5.27
	G8	0	2.66	4.16	5.31	5.01	5.31
	G9	0	2.91	4.36	4.76	5.36	5.01
	G10	0	2.51	4.31	5.16	5.31	5.31
Control	G5	0	0	0	0	3.31	NA
	G6	0	0	0	0	3.06	NA
	G11	0	0	0	0	3.11	NA
	G12	0	0	0	0	3.06	NA

Days post-vaccination (dpc); Days post-challenge (dpc); Not applicable (NA).

VII. Plasmid sequences of un-segmented and segmented PPRV genome

pPPV+GFP Nigeria75/1

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VIII. List of publications

Sections of this thesis work have been published in the following peer-revived journal papers:

Muniraju M, Munir M, Parthiban AR, Banyard AC, Bao J, Wang Z, Ayebazibwe C, Ayelet G, El-Harrak M, Mahapatra M, Libeau G, Batten C and Parida S. 2014a. Molecular Evolution and Emergence of Peste des Petits Ruminants Virus. *Emerging infectious diseases*, http://dx.doi.org/10.3201/eid2012.140684.

Muniraju M, Mahapatra M, Buczkowski H, Batten C, Banyard AC, Parida S. 2014b. Rescue of a vaccine strain of peste des petits ruminants virus: In vivo evaluation and comparison with standard vaccine. *Vaccine*, doi:10.1016/j.vaccine.2014.10.050

Additional publications from this study:

Muniraju M, Mahapatra M, Ayelet G, Babu A, Olivier G, Munir M, Libeau G, Batten C, Banyard AC, Parida S, 2014c. Emergence of lineage IV peste des petits ruminants virus in Ethiopia: Complete genome sequence of an Ethiopian isolate 2010. *Transboundary and Emerging Diseases*. doi:10.1111/tbed.12287.

Muniraju, M., Munir, M., Banyard, A.C., Ayebazibwe, C., Wensman, J., Zohari, S., Berg, M., Parthiban, A.R., Mahapatra, M., Libeau, G., Batten, C., Parida, S., 2014d. Complete Genome Sequences of Lineage III Peste des Petits Ruminants Viruses from the Middle East and East Africa. Genome announcements 2(5):e01023-14. doi:10.1128/genomeA.01023-14.

Muniraju M, El Harrak M, Bao J, Ramasamy Parthiban AB, Banyard AC, Batten C, Parida S, 2013. Complete Genome Sequence of a Peste des Petits Ruminants Virus Recovered from an Alpine Goat during an Outbreak in Morocco in 2008. *Genome Announcements*,1(3):e00096-13. doi:10.1128/genomeA.00096-13.

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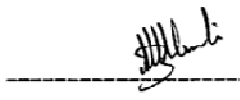
Student's signature:  Date: .....08/03/2016.....