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EPIDEMIOLOGY OF PESTE DES PETITS RUMINANTS VIRUS IN ETHIOPIA AND MOLECULAR STUDIES ON VIRULENCE

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Prof P. DORCHIES	**Président**
Prof P.D. PICAVET	**Directeur de thèse**
Dr P.C. LEFEVRE	**Rapporteur**
Dr S. ZIENTARA	**Rapporteur**
Dr G. LIBEAU	**Membre**
Dr S. BERTAGNOLI	**Membre**

EPIDEMIOLOGY OF PESTE DES PETITS RUMINANTS VIRUS IN ETHIOPIA AND MOLECULAR STUDIES ON VIRULENCE

Abraham Gopilo

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Curriculum Vitae

Abraham Gopilo is born in June 26, 1956 in Kemba in southern Ethiopia. He graduated in 1982 as Doctor of Veterinary Medicine (DVM) from Ukraine Academy of Agriculture, Kiev in the former USSR. He also graduated in Master of Philosophy in Veterinary Science (Veterinary Virology) from Massey University, New Zealand in 1989. He has undertaken short courses (upto three months) in diagnostic virology in 1994 (Maison Alfort, France), 1995 (Pirbright, UK), 1996 (Vienna, Austria). He was in a training visit to Australian National Animal Health Laboratory in Geelong in 1990.

He has a working experience in veterinary clinic of four years (1983-87) and of diagnostic laboratory two years (1990-91) and in the Central Disease Investigation Laboratory (CDIL) which later became National Animal Health Research Center (NAHRC) for ten years (1992-2001). In the laboratory, he worked as National Coordinator for the Pan African Rinderpest Campaign (PARC) serological monitoring and disease surveillance programmes and as National project counter part for the International Atomic Energy Agency (IAEA) and the Ethiopian Science and Technology Commission programmes.

In addition to his assignment in the laboratory he has been a visiting lecturer in Veterinary Faculty in Addis Ababa University (1991-92, 2000) and one time visiting lecturer in tropical veterinary virology in Free University of Berlin in Germany in 2000. He had been successful field supervisor for MSc students (from Zambia and the Sudan) in Free University of Berlin. He had letters of appreciation of remarkable achievement from African Union (AU), Free University of Berlin and International Atomic Energy Agency for expert missions for the Agency in Tanzania (1995), Egypt (1997) and the Sudan (2000).

List of Publications

1. Abraham, G., Sintayehu, A., Libeau G., Albina, E., Roger, F., Laekemariam, Y., Abayneh D., Awoke, K.M (2005) Antibody seroprevalences against peste des petits ruminants (PPR) virus in camels, cattle, goats and sheep in Ethiopia. Preventive Veterinary Medicine 70: 51-57.
2. Abraham, G., Berhan, A. (2001) The use of antigen-capture enzyme-linked immunosorbent assay (ELISA) for the diagnosis of rinderpest and peste des petits ruminants in Ethiopia. Tropical Animal Health and Production 33: 423-30.
3. Abraham, G., Roman, Z., Berhan, A., Huluagerish, A. (1998) Eradication of rinderpest from Ethiopia. Tropical Animal Health and Production 30: 269-72.
4. Abraham, G., Roeder, P., Zewdu, R. (1992) Agents associated with neonatal diarrhea in Ethiopian dairy calves. Tropical Animal Health and Production 24: 74-80.
5. Roeder, P.L., Abraham, G., Kenfe, G., Barrett, T (1994) Peste des petits ruminants in Ethiopian goats. Tropical Animal Health and Production 26: 69-73.
6. Roeder, P.L., Abraham, G., Mebratu, G.Y., Kitching R.P. (1994) Foot-and-mouth disease in Ethiopia from 1988-1991. Tropical Animal Health and Production 26: 163-167.
7. Shiferaw, F., Abditcho, S., Gopilo, A., Laurenson, M.K. (2002) Anthrax outbreak in Mago National Park, southern Ethiopia. Vet Rec. 150: 318-320.
8. Tibbo, M., Woldemeskel, M., Gopilo, A. (2001) An outbreak of respiratory disease complex in sheep in Central Ethiopia. Tropical Animal Health and Production 33: 355-65.

Résumé

La peste des petits ruminants (PPR) est une maladie infectieuse, contagieuse des petits ruminants domestiques ou sauvages. Elle se caractérise par une hyperthermie élevée (autour de 41°C), du jetage, des écoulements oculaires, une stomatite nécrosante, de la diarrhée profuse et généralement une forte mortalité. En Afrique, elle peut avoir différentes incidences cliniques sur les moutons ou les chèvres, depuis l'infection subclinique jusqu'à une infection aiguë létale. En Ethiopie, la PPR clinique est rarement décrite et l'étude de la circulation virale était jusqu'à présent peu développée. Dans ce travail, nous montrons la présence d'anticorps contre le virus de la PPR sur un grand nombre de moutons, chèvres, vaches et chameaux éthiopiens et nous confirmons la transmission naturelle du virus PPR chez ces animaux sans manifestation clinique détectable. Cette absence apparente de pathogénicité peut être liée à une résistance génétique particulière des races de petits ruminants présentes en Ethiopie ou à une variation de la virulence des souches de virus PPR. Afin d'étudier ce dernier point, nous avons entrepris des études in vitro sur des souches isolées en Ethiopie et dans différents pays en comparaison avec une souche vaccinale obtenue par atténuation par passages en série sur culture cellulaire et d'autres souches de morbillivirus.

Dans un premier temps, nous avons testé la capacité du virus PPR à infecter différents systèmes cellulaires. Nous établissons que les cellules VERO (fibroblastes de rein de singe) et 293T (cellules épithéliales de rein humain) permettent la réplication du virus PPR comme celle du virus de la peste bovine. En revanche, les cellules B95a (cellules lymphoblastoïdes B de singe) ne multiplient que le virus de la peste bovine. La capacité d'une cellule à supporter la réplication du virus est de nature à influer son pouvoir pathogène et l'épidémiologie de la maladie. La différence de sensibilité des cellules au virus PPR peut être lié à l'affinité de la glycoprotéine d'enveloppe virale H pour son ou ses récepteurs cellulaires utilisés notamment par le virus de la peste bovine. Pour aborder cette question, nous avons entrepris des comparaisons de séquences

au niveau de la protéine H du virus PPR, en lien avec ce qui a été déjà décrit sur d'autres morbillivirus.

Pour compléter cette étude sur la virulence, nous avons séquencé les promoteurs de plusieurs souches de virus PPR et conduit une analyse des mutations pouvant jouer un rôle dans l'atténuation. En effet, les promoteurs viraux des morbillivirus déterminent la transcription des ARNm viraux et la réplication du génome viral : la modification de leur séquence peut donc affecter leur efficacité et influer sur la virulence de la souche concernée. Nous observons 7 mutations sur les promoteurs de la souche vaccinale du virus PPR en comparaison avec les autres souches virulentes. Certaines mutations sont retrouvées sur les autres morbillivirus, d'autres sont spécifiques du virus PPR. De cette approche moléculaire, nous déduisons également l'intérêt d'utiliser les séquences des promoteurs du virus, relativement très variables par rapport au reste du génome, pour mener des études de phylogéographie et de comparaison entre paramyxovirus.

Le document de thèse a été organisé en 6 chapitres. Le premier concerne l'histoire naturelle de la PPR avec la description du virus, du génome, de l'épidémiologie, de la transmission, des symptômes, de la pathologie, de l'immunologie, du diagnostic, de la lutte contre la maladie et des aspects économiques en Afrique sub-saharienne. Le deuxième chapitre traite de la biologie comparative du virus PPR avec les autres morbillivirus. Le troisième chapitre concerne les travaux d'épidémiologie de la PPR effectués en Ethiopie. Le quatrième volet de ce travail reprend les études sur la spécificité cellulaire du virus PPR et la comparaison des séquences sur la protéine H. Le cinquième chapitre expose les analyses de séquence des promoteurs génomique et antigénomique du virus PPR. Enfin, la dernière partie comprend une discussion générale et des perspectives.

Summary

Peste des petits ruminants (PPR) is an acute and highly contagious viral disease of small ruminants, which is characterised by high fever, ocular and nasal discharge, pneumonia, necrosis and ulceration of the mucuous membrane and inflammation of the gastro-intestinal tract leading to severe diarrhoea and high mortality. In Africa, goats are severely affected while sheep undergo a mild form or rarely suffer clinical disease. PPR is one of the most important economical diseases in Ethiopia. Clinical PPR is confirmed in Ethiopian goats, however, its circulation in other animals has never been described. In the present work, we showed that the antibody seroprevalence in camel, cattle, goat and sheep confirmed natural transmission in these animals without clinical disease. The apparent absence of pathogenicity in these animals may have been due to host resistance or loss of virulence of the virus strain. We have further investigated the latter point by *in vitro* studies on PPRV comparing strains from Ethiopia and other countries with the vaccine strain which has been attenuated after several cell culture passages.

In a first approach, virulence of PPRV was monitored in cell culture system and the use of virus specific monoclonal antibodies enabled to detect differences in virulence between PPRV and RPV. Vero (primate origin) and 293T (human) cell lines supported virus replication permitting the *in vitro* growth of both PPRV and RPV. In contrast to RPV, B95a (marmoset B) cells infected with PPRV were non permissive. The capability of cells to support active virus replication, which may result in intercellular spread and induce damages in infected cells, has implications on the pathogenesis and epidemiology. Cellular receptors are major determinants of host range and tissue tropism of a virus. The difference in infectivity of PPRV and RPV may have depended on the H protein epitopes and their cellular receptors. Therefore, we decided to compare the amino acid epitope of H protein of PPRV with that of other morbilliviruses.

As part of our investigation of virulence factors, we have sequenced and compared genome and antigenome promoters of a vaccine strain with field strains of PPRV. The promoters contain the polymerase binding sites to initiate and generate the positive-strand replication and transcription of mRNAs. Nucleotide base change differences between vaccine strain and field strains would provide molecular basis for attenuation. Alignment of the genome promoter sequences revealed seven nucleotide mutations at certain positions. Our finding on nucleotide mutation on PPRV are in agreement with the nucleotide changes in rinderpest virus and other morbillivirus promoter regions between vaccine strain and wild type virus. Certain mutations were specific to PPRV. The promoter sequences were clustered around the geographic origin of the viruses and were lineage specific. Phylogenetic analysis of PPRV promoters was used for PPR phylogeograhy, and for comparison with other paramyxoviruses.

The thesis is divided in 6 chapters. The first chapter deals with the natural history of PPR including the virus, the genome, epidemiology, transmission, clinical signs, immunology, diagnosis, control and its economic cost in the low income subsistence farming systems in sub-Saharan Africa. The second chapter is about comparative biology of PPRV with regard to other groups of morbillivirus genus in the *Paramyxoviridae* family.

The third chapter deals with field study and observations on epidemiology of PPR in Ethiopia. In chapter four, PPRV virulence was monitored in cell culture system and comparison of H protein epitopes. In chapter five, sequence analysis of genome and antigenome promoters of PPRV was described In chapter six, general discussion and recommendations were forwarded.

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List of Abbreviation

AGID	Agar gel immunodiffusion
AGP	Antigenome promoter
ATCC	American type cell culture
cDNA	Complementary deoxiribonucleic acid
CDV	Canine distemper virus
C-ELISA	Competitive enzyme –linked immunosorbent assay
CIEP	Counter immunoelectrophoresis
CMV	Cetacean morbillivirus
CPE	Cytopathic effects
DEPC	Diethyl pyrocarbonate
DMEM	Dulbeco's minimum essential medium
DMV	Dolphin morbillivirus
EDI	ELISA data information
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cells sorter
FAO/GREP	Food and Agriculture organization/Global Rinderpest Eradication Project
FAO/IAEA	FAO/ International Atomic Energy Agency
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GDP	Gross domestic product
GP	Genome promoter
HRPO	Horseradish peroxidase

IC-ELISA	Immuno capture enzyme-linked immunosorbent assay
IFAT	Indirect fluorescent antibody test
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ISCOM	Immune stimulating complex
MAbs	Monoclonal antibodies
MEM	Minimum essential medium with Earle's salts
MIBE	Measles inclusion body encephalitis
MOCL	monocyte derived cells lines (cloned sheep origin)
MOI	Multiplicity of infection (a proportion of cell/ml to virus, TCID50)
mRNA	Messenger ribonucleic acid
MV	Measles virus
NPV	net present value
O.I.E.	Office International des Epizooties
OD	optical density
ODE	Old dog encephalitis
ORF	open reading frame
PANVAC	Pan African Veterinary Vaccine Centre
PARC	Pan African Rinderpest Campaign
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood monocyte cells
PBS	Phosphate-buffered saline
PBST	PBS plus 0,05% Tween 20

PCR	Polymerase chain reaction
PCV	Packed cell volume
PDV	Phocine distemper virus
PMV	Porpoise morbillivirus
PPR	Peste des petits ruminants
PPRV	Peste des petits ruminants virus
RACE	Rapid amplification of cDNA ends
RBC	Red blood cells
RBOK	Rinderpest bovine old Kabete
RBT/1	Reedbuck/1 strain of rinderpest isolated in Kenya in 1960s
RNA	ribonucleic acid
RP	Rinderpest
RPV	Rinderpest virus
RT/PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLAM	Signaling Lymphocyte Activation Molecules (CD150)
SSPE	Subacute sclerosing panencephalitis
TBE	Tris-borate-EDTA
TCID 50	Tissue culture infectious dose fifty
TCRV	Tissue culture rinderpest vaccine
Vero cells	African green monkey kidney cells
VNT	Virus neutralization test
VV	Vaccinia virus

Chapter 1.

Review of Literature

1.1. Introduction

For centuries, morbillivirus infections have had a huge impact on both human beings and animals. Morbilliviruses are highly contagious pathogens that cause some of the most devastating viral diseases of humans and animals worldwide (Murphy *et al*., 1999). They include measles virus (MV), canine distemper virus (CDV), rinderpest virus (RPV), and peste des petits ruminants virus (PPRV). Furthermore, new emerging infectious diseases of morbilliviruses with significant ecological consequences for marine mammals have been discovered in the past decade. Phocid distemper virus (PDV) in seals and the cetacean morbillivirus (CMV) have been found in dolphins, whales and porpoises (Barrett *et al*., 1993, Domingo *et al*., 1990, McCullough *et al*., 1991).

The great cattle plagues of the 18th and 19th centuries in Europe were introduced by traders from the East (Wilkinson, 1984). Subsequently, rinderpest was introduced into Africa from India during colonial wars in Abyssinia in the 1890s, with devastating effects on the susceptible domestic and wildlife species (Mack, 1970). International campaigns are under way to eradicate globally both MV and RPV. Peste des petits ruminants virus (PPRV), originally endemic in west Africa has spread across East Africa, the Middle East and southern Asia as far as Bangladesh (Shaila *et al*., 1996) and Turkey (Ozkul *et al*., 2002).

Morbilliviruses are enveloped, nonsegmented negative strand RNA viruses and constitute a genus within the family *Paramyxoviridae.* They cause fever, coryza, conjunctivitis, gastroenteritis, and pneumonia in their respective host species. The major sites of viral propagation are lymphoid tissues, and acute diseases are usually accompanied by profound lymphopenia and

immunosuppression, leading to secondary and opportunistic infections (Appel and Summers, 1995; Murphy *et al*., 1999).

1.2. History

Peste des petits ruminants (PPR) is a highly contagious and infectious viral disease of domestic and wild small ruminants (Furley *et al*., 1987). It is an economically significant disease of small ruminants such as sheep and goats (Dhar *et al*., 2002). It was first described in Côte d'Ivoire in West Africa (Gargadennec and Lalanne, 1942) where it used to be named as Kata, psuedo-rinderpest, pneumoenteritis complex and stomatitis-pneumenteritis syndrome (Braide, 1981). Investigators soon confirmed the existence of the disease in Nigeria, Senegal and Ghana. For many years it was thought that it was restricted to that part of the African continent until a disease of goats in the Sudan, which was originally diagnosed as rinderpest in 1972, was confirmed to be PPR (Diallo, 1988). The realization that many of the cases diagnosed as rinderpest among small ruminants in India may, instead, have involved the PPR virus, together with the emergence of the disease in other parts of western and South Asia (Shaila *et al*., 1996), signified its ever-increasing importance. It has received a growing attention because of its wide spread, economic impacts (Lefèvre and Diallo, 1990) and the role it plays in complication of the ongoing global eradication of rinderpest and epidemiosurveillance programmes (Couacy-Hymann *et al*., 2002).

1.3. Causative Agent:

PPR is caused by a virus that was assumed for a long time to be a variant of rinderpest adapted to small ruminants. However, studies based on virus cross neutralization and electron microscopy showed that it was a morbillivirus that had the physicochemical characteristic of a distinct virus though biologically and antigenically related to RPV. It was also shown to be an immunologically distinct virus with a separate epizootiology in areas where both viruses were enzootic (Taylor, 1979a). The development of specific nucleic acid probes for hybridisation

studies and nucleic acid sequencing have proved that PPR virus is quite distinct from rinderpest virus (Diallo *et al*., 1989a). PPRV is in the *Morbillivirus* genus of the *Paramyxoviridae* family (Gibbs *et al*., 1979). The *Morbillivirus* genus also includes other six viruses: measles virus (MV), rinderpest virus (RPV), canine distemper virus (CDV), phocine morbillivirus (PMV), porpoise distemper virus (PDV) and dolphin morbillivirus (DMV) (Barrett *et al*., 1993a, Barrett, 2001).

1.3.1. Virus structure and genome organization

When viewed through electronmicroscope, morbilliviruses display the typical structure of *Paramyxoviridae*: a pleomorphic particle with a lipid envelope which encloses a helical nucleocapsid (Gibbs *et al*., 1979). The nucleocapsids have a characteristic herring-bone appearance. Morbilliviruses are linear, non-segmented, single stranded, negative sense RNA viruses with genomes approximately 15–16 kb in size and 200 nm diameter (Norrby and Oxman, 1990). Full length genome sequences are available for MV (Cattaneo *et al*., 1989), RPV (Baron and Barrett, 1995), CDV (Barrett *et al*., 1987), PPRV (Bailey *et al*., 2005) and the dolphin morbillivirus (DMV) (Rima *et al*., 2003). These data have been used to establish reverse genetics, a technology critical for negative sense RNA virus research (Nagai, 1999; Neumann *et al*., 2002). The sequence data show striking similarities and it is believed that the morbilliviruses have an identical genome organization (Barrett *et al*., 1991; Banyard *et al*., 2005). The genome is divided into six transcriptional units (Fig. 1-1, Fig. 1-2) encoding two non structural (V and C protein) and six structural proteins (Barrett, 1999; Baron and Barrett, 1995; Diallo, 1990). The gene order has been established as follows 3'-N-P/C/V-M-F-H-L-5' (Barrett, 1999; Barrett *et al*., 1991; Diallo, 1990). The genome sequence was divisible by six, a feature shared with other *Paramyxoviridae* (Calain and Roux, 1993). The exact length of morbillivirus genomes differs owing to a variable size of the junction between the M and F genes, but not because of varied protein lengths. This junction has a particularly high GC content (65%) but no obvious role in replication has been shown (Liermann *et al*., 1998; Radecke *et al*., 1995).

The nucleocapsid (N) protein: The N protein is the most abundant viral protein both in the virion and in infected cells (Diallo *et al*., 1987). It directly associates with the RNA genome to form the typical herring bone structure of morbillivirus nucleocapsids. There is a single transcription promoter at the 3' end, upstream to the first codon of the N gene, including the non coding part of the N gene and a 52-56 bases leader sequence (Billeter *et al*., 1984, Crowley *et al*., 1988, Ray *et al*., 1991). Various roles have been proposed for the leader RNA, including RNA binding site for the polymerase to initiate and generate positive strand RNA replication (Fig. 1-3) (Norrby and Oxman, 1990; Walpita, 2004), and down regulation of host cell transcription (Ray *et al*., 1991). Transcription and replication of morbilliviruses are controlled by untranslated regions (UTRs) at the 3' and 5' ends of the genome, known as the genome (GP) and antigenome (AGP) promoters (Lamb and Kolakofsky, 2001). In PPRV, these are represented by nucleotides 1–107 and 15840–15948, respectively. A conserved stretch of 23–31 nucleotides at the 3' terminus of both the GP and the AGP has been shown to be an essential domain required for promoter activity. The sequence of the promoters was highly conserved in PPRV (Mioulet *et al*., 2001). Conserved sequences at the junction between the GP and the N gene start, which includes the intergenic triplet, are also required for transcription (Mioulet *et al*., 2001). The intergenic regions are made up of four elements: a semi conseved polyadenylation signal, a highly conserved GAA sequence, a semi conserved start signal for the next gene and variable length of 5' and 3' untranslated regions (UTRs) (Barrett *et al*., 1991).

A poly U tract which, is responsible for polyadenylation of the positive sense transcripts produced by the viral RNA-dependent RNA polymerase, was located 52 bases downstream of the N ORF stop. This sequence is highly conserved in the morbilliviruses and acts as part of a gene stop and polyadenylation signal. Reduction in size of the poly U tract of the paramyxovirus simian virus 5 (SV5) from six residues to four was shown to diminish downstream initiation to 20–30% of wild-type levels indicating a possible role as a critical spacer region between gene

stops and starts (Rassa *et al*., 2000). This sequence was maintained throughout the genome of PPRV except at the M/F, and F/H junctions where the U tract is interrupted by a G residue. It is unknown if this G insertion has any modulating effect on the polymerase stuttering mechanism employed to polyadenylate nascent mRNAs, or on the transcription of the downstream mRNA(s). Immediately following the poly U tract was a conserved triplet (GAA) that has been shown to be an intergenic region which is not transcribed during mRNA synthesis but which is an essential signal for the activity of the viral polymerase since mutations or deletions in this region can reduce or abrogate viral replication (Kolakofsky *et al*., 1998). Deletion mutagenesis studies indicate that the 5' UTRs for CDV and RPV F genes may serve to direct translation initiation from a particular AUG, thus ensuring efficient translation of F protein. (Evans *et al*., 1990). The rate of transcription of mRNAs from each gene is proportional to its distance from the genome promoter, since there is a chance that at each intergenic junction the polymerase may detach from the template and reinitiate transcription from the 3' end (Barrett *et al*., 1991).

The second transcription unit encodes the P, C and V proteins. The P protein of morbilliviruses interacts with both the N and L proteins to form the viral polymerase. The N terminus of V is identical to P but polymerase slipping at the editing site can result in a frame shift whereby an inserted G residue in the mRNA directs the production of an alternative C terminus (Cattaneo *et al*., 1989; Wardrop and Briedis, 1991, Mahapatra *et al*., 2003). The hexamer phasing of the P gene editing site is also thought to play a critical role in this process (Kolakofsky *et al*., 1998). The alternative C terminus of PPRV has seven conserved cysteine residues that are thought to interact to form a motif for binding metal ions (especially zinc). This was shown experimentally for both Sendai virus-5 (SV5) and MV V proteins (Paterson *et al*., 1995). The C and V proteins of paramyxoviruses, although essentially non-structural, have been shown to have critical roles in infection. In RPV they were shown to be important for replication (Baron and Barrett, 2000). C-minus mutants showed growth defects in vitro, this being related to a reduced level of mRNA

transcription. In contrast V-minus mutants were not defective in vitro, but had an altered cytopathic effect and increased genome/antigenome RNA production. The C and V proteins of paramyxoviruses also act as interferon antagonists, modifying the cellular immune response to infection (Gotoh *et al*., 2001; Horvath, 2004).

The Matrix (M) proteins are basic membrane associated molecules that interact with surface glycoproteins in the lipid envelope as well as the virion RNP. They are the most highly conserved proteins in the *Paramyxovirus* family and this was reflected in the conservation of the PPRV M protein when compared to that of other morbilliviruses. This high conservation is probably due to the pivotal role the M protein plays in virion morphogenesis. A small protein with such a critical role is likely to be intolerant to variation, especially within a genus whose members are antigenically so similar. It is a non-glycosylated envelope protein thought to be involved in nucleocapsid-envelope recognition and envelope formation during the budding process of virions from the host cell membrane (Kingsbury, 1990). M interacts with both the nucleocapsid and the cytoplasmic tails of the F and H glycoproteins.

The F protein is also highly conserved. Paramyxoviruses generate an inactive precursor (F0) which is cleaved by host cell enzymes to yield an active di-sulphide linked protein F1–F2 and the cleavage site was also conserved (Lamb and Kolakofsky, 2001). The F protein is one of two glycosylated envelope proteins that constitute the peplomers or surface projections. Synthesized as a precursor, F_0 is subsequently cleaved by cellular proteases into two disulfide-linked polypeptides, F_1 and F_2 (Sato *et al*., 1988). Proteolytic cleavage is believed to be essential for F protein biologic activity.

The H protein is responsible for attachment of the virus to the host cell (Choppin and Scheid, 1980, Lamb and Kolakofsky, 2001). The biological activity of the H protein is one of the criteria for classification of *Paramyxoviridae*. H proteins are highly variable (Blixenkrone- Moller *et al*., 1996). Indeed, along with the P, the H is the least conserved of the morbillivirus proteins. The H

protein is the least conserved among CDV, RPV and MV (37% identity between CDV and MV) (Blixenkrone-Moller *et al*., 1996) and 37% amino acid homology between MV and CDV (Wild *et al*., 1995). Members of the *Paramyxovirus* genus (e.g. Newcastle disease virus) have H protein with both hemagglutinating and neuraminidase activities (Scheid and Choppin, 1974); the *Morbillivirus* H protein exhibits only hemagglutinating activity, and the H protein of members of *Pneumovirus* genus (Respiratory syncytial viruses) has neither hemagglutinating nor neuraminidase activities (Kingsbury *et al*., 1978).

The large (L) protein is the enzymatic component of the viral transcriptase and replicase. The L proteins are multi-functional and, in addition to their polymerase activity, have methylation, capping and polyadenylation activities (Lamb and Kolakofsky, 2001). Morbillivirus L proteins have three highly conserved domains (designated A, B and C), separated by two hinge regions which vary greatly between morbilliviruses (McIlhatton *et al*., 1997). The conservation of the D and N residue in this motif is known to be an absolute requirement for polymerase activity (Chattopadhyay *et al*., 2004). Specifically, the GD residues of this motif constitute part of a turn structure that is predicted to be the core polymerase motif (Poch *et al*., 1990). This protein region is involved in nucleic acid binding and is formed when leucine residues, from adjacent alpha-helices, interdigitate to stabilise the L protein (Ramji and Foka, 2002; Vinson *et al*., 1989).

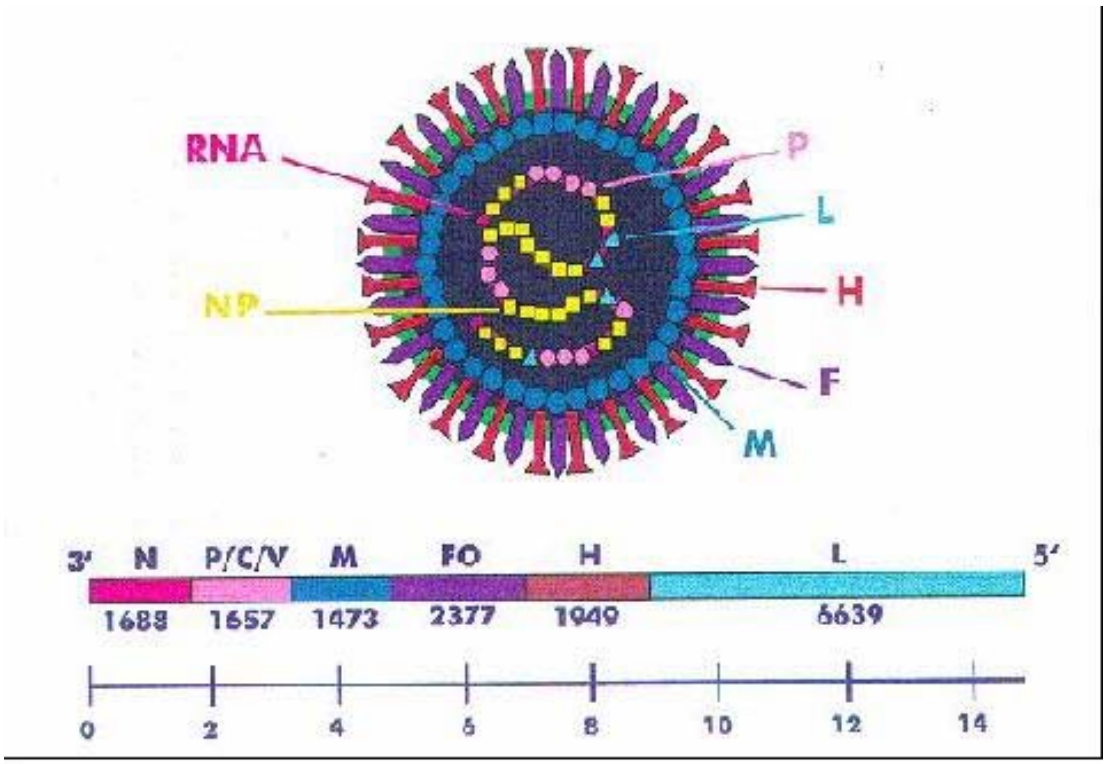

Fig. 1-1 Genome of Morbilliviruses. (image, anonym)

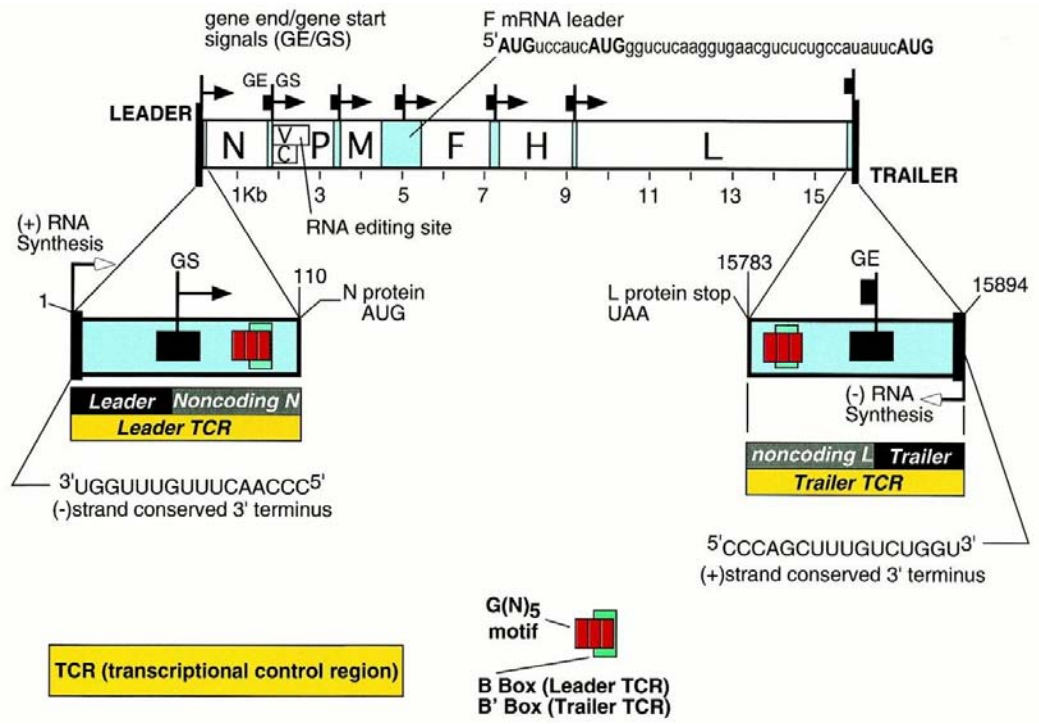

Fig. 1-2. Genes and promoters of *Morbillivirus* (from Parks *et al*, 2001): the protein coding regions (N, P, V, C, M, F, H, and L), noncoding intergenic regions and the leader and trailer regions along with specialized sequence motifs are shown. The genome promoter includes the leader sequence and the non coding regions N at the 3' end of the genomic RNA. The antigenome promoter includes the trailer sequence and the untranslated regions of the L gene at 5' end. Gene start (GS) and gene end (GE), enclosing the intergenic trinucleotide motifs are also shown.

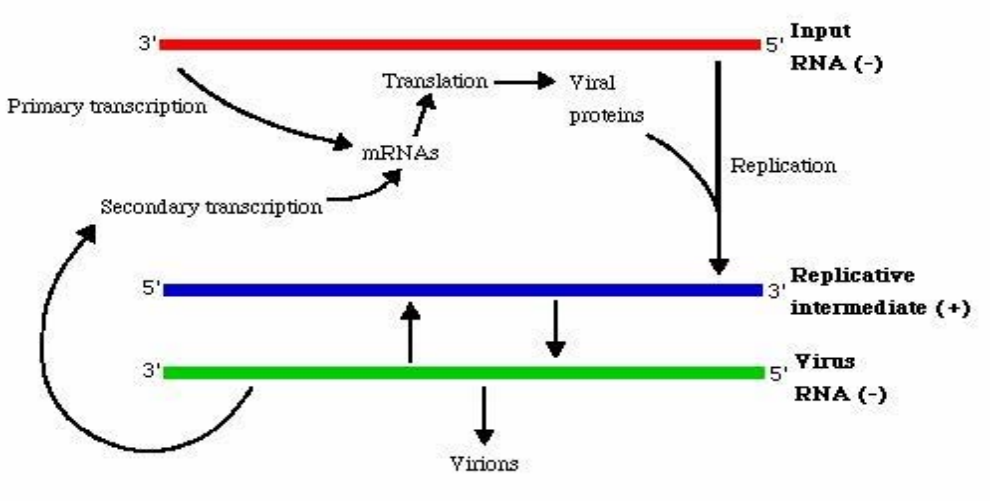

Fig. 1-3. *Morbillivirus* replication pathway. Full-length genome copies are made via replication intermediate strands of the minigenome. (image, anonym, unpublished)

1.4. Geographical Distribution:

PPR is known to be present in a broad belt of sub-Saharan Africa, Arabia, the Middle East and Southern Asia. Major outbreaks in Turkey and India in recent years have indicated a marked rise in the global incidence of PPR (Nanda *et al*., 1996; Ozkul *et al*., 2002; Shaila *et al*., 1996).

The virus was isolated in Nigeria (Taylor and Abegunde, 1979), Sudan (ElHag and Taylor, 1984), Saudia Arabia (Abu Elzein *et al*., 1990), India (Shaila *et al*., 1989, Nanda *et al*., 1996) and Turkey (Ozkul *et al*., 2002). Serological evidences were detected in Syria, Niger and Jordan, while the virus presence was confirmed with cDNA probe in Ethiopia (Roeder *et al*., 1994) and Eritrea (Sumption *et al*., 1998), respectively. Genetic relationship between PPR viruses isolated from different geographical regions was studied by sequence comparison of the F-protein gene. Four lineages were revealed (Shaila *et al*., 1996, Dhar *et al*., 2002) (Fig. 1-4, Fig. 1-5). Lineage 1 is represented by viruses isolated in Africa in 1970s (Nigeria/1975/1, Nigeria/1975/2, Nigeria1975/3, Nigeria/1976/1 and Senegalese strain). Lineage 2 which includes viruses isolated in the late 1980s in West Africa (Ivory Coast and Guinea) is the only African lineage that did not cross the Red Sea to the Asian countries. Lineage 3 is a combination of isolates from Sudan (Meilig /1972) (Diallo, 1988), Ethiopia (Roeder *et al*., 1994). Lineage 4 of PPR virus isolates which includes the Asian isolates from Israel/1994, Iran/1994, Nepal/1995, Bangeldesch/1993 and India (Shaila *et al*., 1996), is confined to Asia. Recently, it was reported in Turkey (Ozkul *et al*., 2002). The presence of the two African lineages in Asia beside a distinct Asian lineage may be taken as indication of the trade route of spread of the disease.

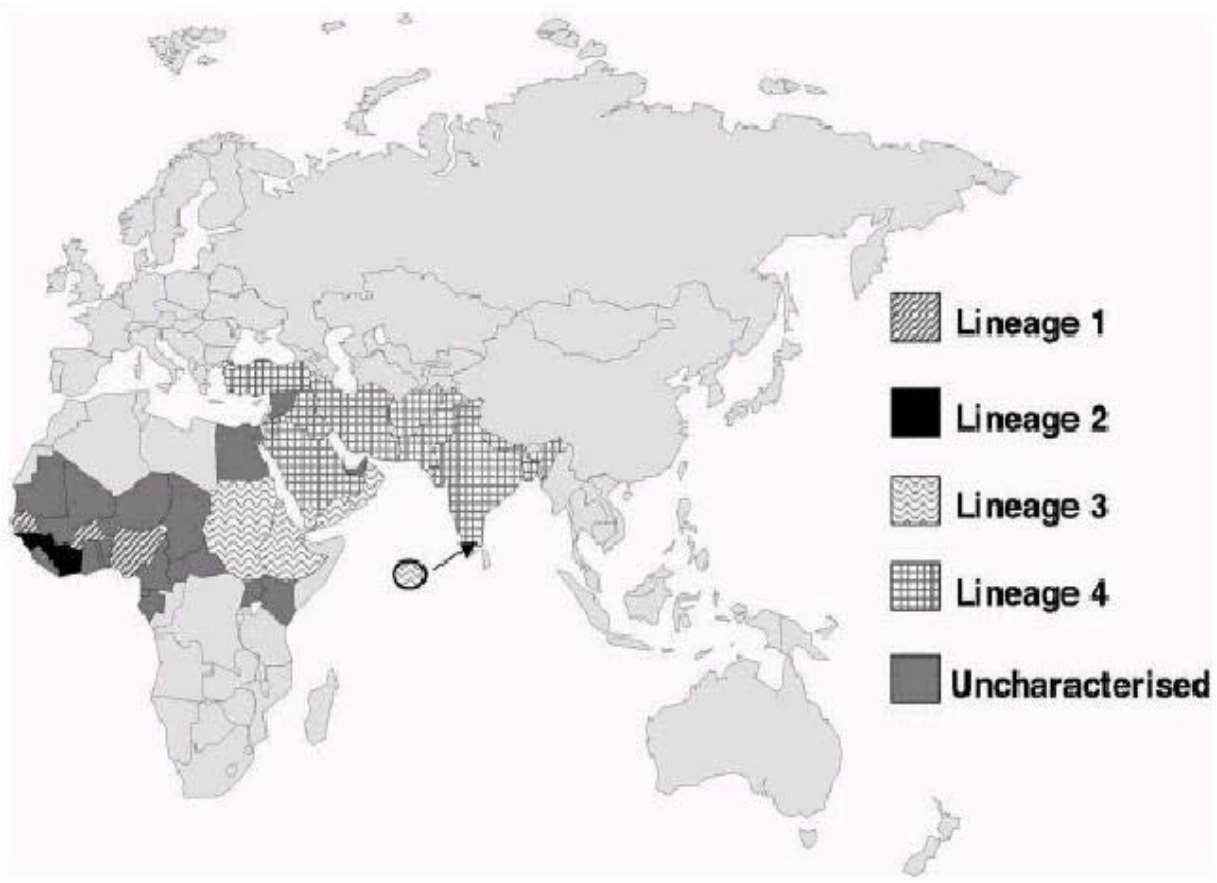

Fig. 1-4 Geographic distribution of PPRV lineages (Dhar *et al*., 2002)

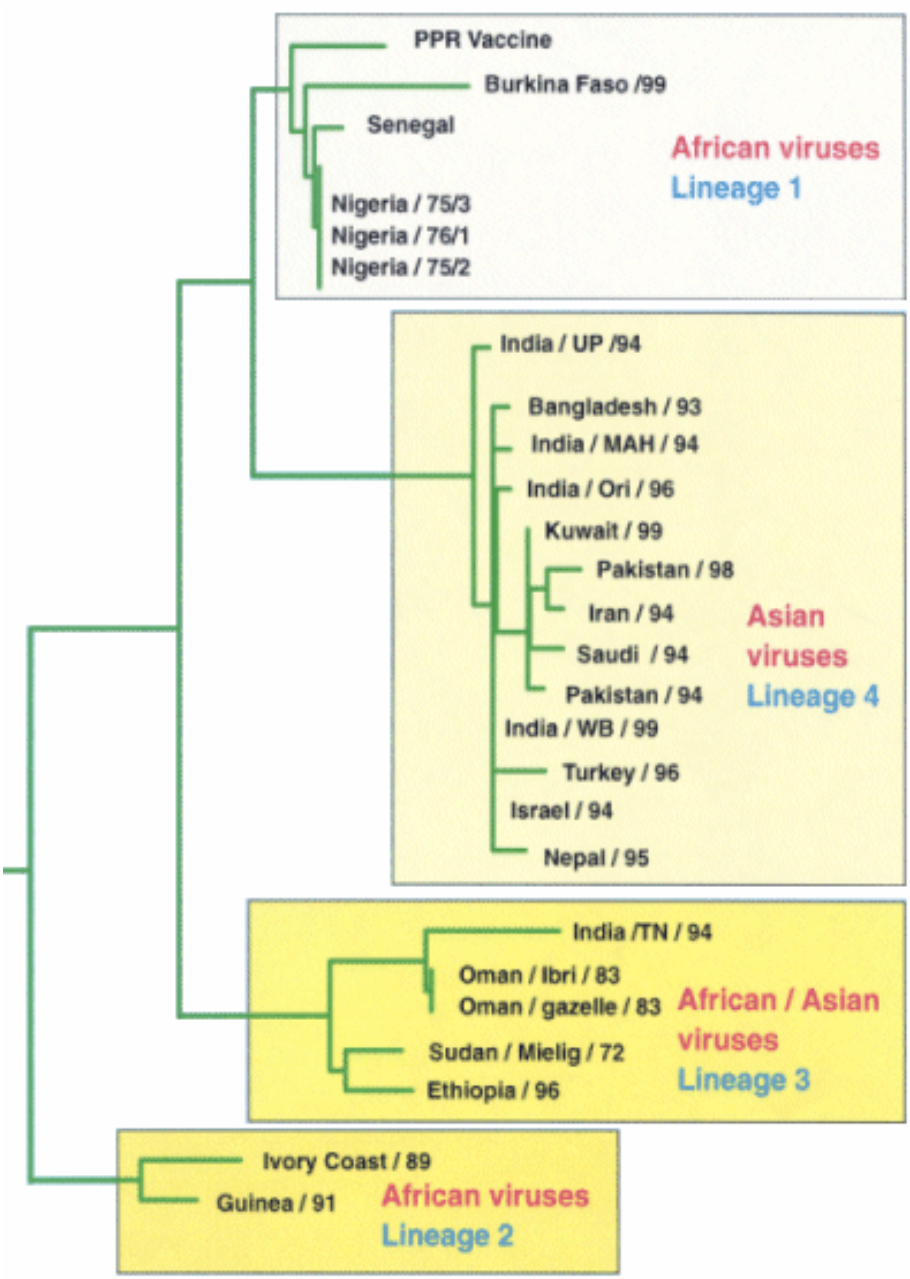

Fig. (1-5) Phylogenetic relationships of the Peste des petits ruminants virus isolates based on (F) protein gene (Ozkul *et al*., 2002)

1.5. Epidemiology

1.5.1. Transmission:

Transmission requires close contact between infected animals in the febrile stage and susceptible animals (Braide, 1981) because of the lability of the virus outside the living host. The discharges from eyes, nose and mouth, as well as the loose faeces, contain large amounts of the virus. Fine infective droplets are released into the air from these secretions and excretions, particularly when affected animals cough and sneeze (Bundza *et al*., 1988; Taylor, 1984). Animals in close contact inhale the droplets and are likely to become infected. Although close contact is the most important way of transmitting the disease, it is suspected that infectious materials can also contaminate water and feed troughs and bedding, turning them into additional sources of infection. These particular hazards are, however, probably fairly short-term since the PPRV, like rinderpest, would not be expected to survive for long outside the host. Indirect transmission seems to be unlikely in view of the low resistance of the virus in the environment and its sensitivity to lipid solvent (Lefèvre and Diallo, 1990). There is no known carrier state for PPRV. Trade in small ruminants, at markets where animals from different sources are brought into close contact with one another, affords increased opportunities for PPR transmission, as does the development of intensive fattening units.

1.5.2. Host Range and pathogenicity:

PPR is mainly a disease of small ruminants. It affects goats and sheep. PPR virus exhibits different levels of virulence between sheep and goats. Goats are severely affected while sheep generally undergo a mild form (Lefèvre and Diallo, 1990). Although infected, sheep rarely suffer clinical disease (El Hag Ali and Taylor, 1988; Roeder *et al.,* 1994). An outbreak with a high mortality in sheep was reported by Taylor (1984) who hypothesised that sheep possessed an innate resistance to the clinical effects of disease, but occasional field strains could overcome this resistance and produce high mortality (Taylor, 1984). Breed may affect the outcome of PPR virus

infection and its epidemiology, the Guinean breeds (West African dwarf, Iogoon, kindi and Djallonke) are known to be highly susceptible (Lefèvre and Diallo, 1990). This is in agreement with the finding that British breed exhibited severe clinical reaction when infected experimentally while the Sudanese breeds failed to develop a characteristic clinical response (El Hag and Taylor, 1984). A more recent observation detected variations in breed susceptibility within goats in West Africa. The acute form of the disease was observed in WAD goats while WALL breed developed only mild form (Diop *et al.*, 2005). (Fig. 1-6 and Fig. 1-7).

Fig. 1-6 PPR resistance goat breeds in sahelian region (Photo by Dr V. Martin)

Fig. 1-7 PPR resistance sheep in West Africa (Photo, anonym, Dakar, Senegal)

In India and the Middle East both goats and sheep are affected with equally devastating consequences. In India, morbidity and case fatality reach 10 and 25% respectively in flocks of indigenous sheep (Shaila *et al*., 1989). The outbreak will not involve cattle, whether rinderpest vaccinated or not, even if they are in contact with affected goats and sheep. Cattle and pigs are known to be a dead end host and all attempt to induce clinical disease in adult cattle experimentally failed; they undergo a silent or subclinical infection that protect them against subsequent challenge with virulent strain of RP (Gibbs *et al*., 1979; Taylor, 1984). Sero-neutralization test for the presence of PPR antibodies detected 4.2% in 142 camels (Ismail *et al*., 1995). PPR affect wildlife animals both under field condition and experimentally. The disease was induced experimentally in American white deer *(Odocoileus virginianus*) which was found to be susceptible (Hamdy *et al*., 1976) and a field outbreak was reported from a zoological collection in Alain (Furley *et al*., 1987). It caused a high mortality and severe disease in Dorcas Gazelles (*Gazella dorcas*), Nubian Ibex (*Capra ibex nubiana*), Laristan sheep (*Ovis orientalis laristani*) and gemsbok (*Oryx gazellaa*). Subclinical involvement of Nigale (*Tragelaphinae*) was suspected. In another report from Saudi Arabia, PPR was suspected on clinical and serological base in Gazaelle and deer (Abu Elzein *et al*., 1990). Antelope and other small wild ruminant species can also be severely affected (Abu Elzein *et al*., 2004).

1.5.3. Pattern of the disease:

In general, a morbidity is common, particularly in fully susceptible goat populations. Milder forms of the disease may occur in sheep and partially immune goat populations. There are considerable differences in the epidemiological pattern of the disease in the different ecological systems and geographical areas. In the humid Guinean zone where PPR occurs in an epizootic form, it may have dramatic consequences with morbidity of 80%-90% accompanied with a mortality between 50 and 80% (Lefèvre and Diallo, 1990). While in arid and semi-arid regions, PPR is seldomly fatal but usually occurs as a subclinical or inapparent infection opening the door for other infections such as Pasteurellosis (Lefèvre and Diallo, 1990). Though outbreaks in West Africa coincide with the wet rainy season, Opasina and Putt (1985) observed outbreaks during the dry season in two different ecological zones. A high morbidity of 90% accompanied with 70% case fatality was reported from Saudi Arabia (Abu Elzein *et al*., 1990).

Serological data from Nigeria revealed that antibodies occur in all age groups from 4-24 months indicating a constant circulation of the virus (Taylor, 1979b). In Oman the disease persisted on a year round basis maintaining itself in the susceptible yearling population (Taylor *et al*., 1990). Therefore, an increase in incidence reflects an increase in number of susceptible young goats recruited into the flocks rather than seasonal upsurge in the virus activity, since its upsurge pend on the peak of kidding seasons (Taylor *et al*., 1990). Moreover, the susceptibility of young animals aged 3 to 18 months was proved to be very high, being more severely affected than adults or unweaned animals (Taylor *et al*., 1990).

1.6. Clinical Signs

Clinical signs of PPR have been well documented (Hamdy *et al*., 1976; Obi, 1984; Lefèvre, 1987; Taylor, 1984; Bundza *et al*., 1988; Roeder *et al*., 1994; Roeder and Obi, 1999). Introduction of PPR into a flock may be associated with any of the following:

- history of recent movement or gathering together of sheep and/or goats of different ages with or without associated changes in housing and feeding;
- introduction of recently purchased animals; contact in a closed/village flock with sheep and/or goats that had been sent to market but returned unsold;
- change in weather such as the onset of the rainy season (hot and humid) or dry, cold periods, contact with trade or nomadic animals through shared grazing, water and/or housing;
- a change in husbandry (e.g. towards increased intensification) and trading practices.

Following infection there is a 3–4 day incubation period during which the virus replicates in the draining lymph nodes of the oro-pharynx before spreading via the blood and lymph to other tissues and organs including the lungs causing a primary viral pneumonia. The predominant form of the disease is the acute form. The salient clinical signs start with sudden rise in body temperature to 39.5 - 41°C. Affected animals breathe fast, sometimes so fast that they exhibit rocking movements with both the chest and abdominal walls moving as the animal breathes. Severely affected cases show difficult and noisy breathing marked by extension of the head and neck, dilation of the nostrils, protrusion of the tongue and soft painful coughs. They have obvious signs of pneumonia. A clear watery discharge starts to issue from the eyes, nose and mouth, later becoming thick and yellow as a result of secondary bacterial infection. Appearance of a serous to mucopurulent nasal discharge which may crust over and occlude the nostril, sneezing, ocular discharge resulting in matting of the eyelids. The discharges wet the chin and

the hair below the eye; they tend to dry, causing matting together of the eyelids, obstruction of the nose and difficulty in breathing (Fig. 1-8). Unlike RP, there is a definite but inconstant, respiratory system component (Brown *et al*., 1991; Bundza *et al*., 1988). One to two days after fever has set in, the mucous membranes of the mouth and eyes become very reddened. Then, epithelial necrosis causes small pin-point greyish areas on the gums, dental pad, palate, lips, inner aspects of the cheeks and upper surface of the tongue. These areas increase in number and size and join together. The lining of the mouth is changed in appearance. It becomes pale and coated with dying cells and, in some cases, the normal membrane may be completely obscured by a thick cheesy material. Underneath the dead surface cells, there are shallow erosions. Gentle rubbing across the gum and palate with a finger may yield a foul-smelling material containing shreds of epithelial tissue (Braide, 1981) (Fig. 1-9). Body temperature usually remains high for about 5-8 days, and then slowly returns to normal prior to recovery or drops below normal before death (Fig. 1-10).

Fig. 1-8 Clinical signs, discharges (Photo, Abraham and Roeder, Ethiopia)

Fig. 1-9 Mouth lesions (Photo, Abraham and Roeder, Ethiopia)

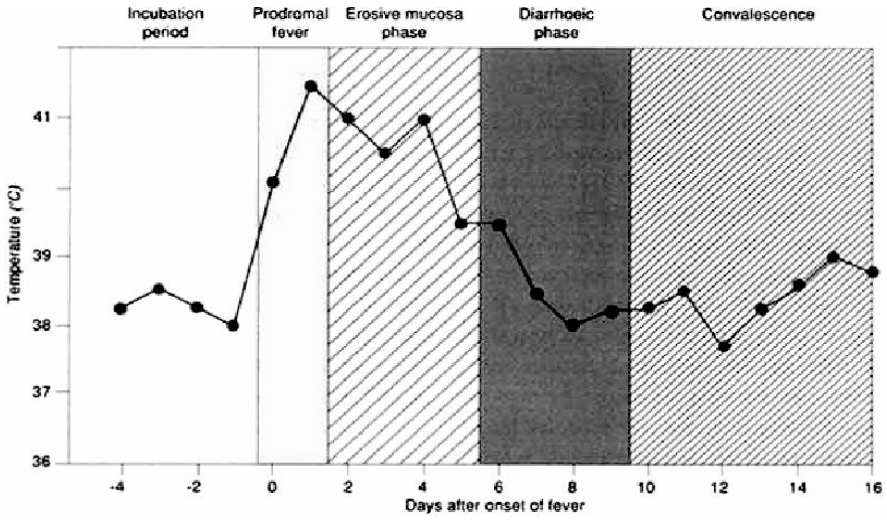

Fig. 1-10 Body temperature fluctuation and phases of clinical disease (Scott *et al.*, 1986)

Diarrhoea commonly appears about two to three days after the onset of fever although, in early or mild cases, it may not be obvious. The faeces are initially soft and then watery, foul-smelling and may contain blood streaks and pieces of dead gut tissue. Where diarrhoea is not an obvious presenting sign, the insertion of a cotton wool swab into the rectum may reveal evidence of soft faeces which may be stained with blood. Such victims may eventually become dehydrated with sunken eyeballs, and death often follows within seven to ten days from onset of the clinical reaction. Other animals will recover after a protracted convalescence.

The affected animals had lymphocytopenia, elevated PCV (above 60% while normal 35-45%), very high RBCs count while the level of hemoglobin and the white blood cells was normal (Furley *et al*., 1987). A common feature in later stages of the sub-acute disease is the formation of small nodular lesions in the skin on the outside of the lips around the muzzle. The exact cause of this is not known.

1.7. Pathology

Pathogenesis: PPR virus, like other morbilliviruses, is lymphotropic and epitheliotropic (Scott, 1981). Consequently, it induces the most severe lesions in organ systems rich in lymphoid and epithelial tissues. The respiratory route is the likely portal to entry. After the entry of the virus through the respiratory tract system, it localizes first replicating in the pharyngeal and mandibular lymph nodes as well as tonsil. Viremia may develop 2-3 days after infection and 1-2 days before the first clinical sign appears. Subsequently viremia results in dissemination of the virus to spleen, bone marrow and mucosa of the gastro-intestinal tract and the respiratory system (Scott, 1981).

Post mortem findings: The carcass of an affected animal is usually emaciated, the hindquarters soiled with soft/watery faeces and the eyeballs sunken. The eyes and nose contain dried-up discharges. Lips may be swollen; erosions and possibly scabs or nodules in late cases. The nasal cavity is **c**ongested (reddened) lining with clear or creamy yellow exudates and erosions. They

may be dry with erosions on the gums, soft and hard palates, tongue and cheeks and into the oesophagus. The lung is dark red or purple with areas firm to the touch, mainly in the anterior and cardiac lobes (evidence of pneumonia). Lymph nodes (associated with the lungs and the intestines) are soft and swollen. Abomasum congested with lining haemorrhages.

The pathology caused by PPR is dominated by necrotizing and ulcerative lesions in the mouth and the gastro-intestinal tract (Roeder *et al*., 1994). Erosion in the oral cavity is a constant feature. The rumen reticulum and omasum rarely exhibit lesions. Occasionally, there may be erosions on the pillars of the rumen. The omasum is a common site of regularly outlined erosions often with oozing blood. Lesions in the small intestine are generally moderate, being limited to small streaks of hemorrhages and, occasionally, erosions in the first portions of the duodenum and the terminal ileum. The large intestine is usually more severely affected, with congestion around the ileo-cecal valve, at the ceco-colic junction and in the rectum. In the posterior part of the colon and the rectum, discontinuous streaks of congestion “zebra stripes” form on the crests of the mucosal folds.

In the respiratory system, small erosion and petechiae may be visible on the nasal mucosa, turbinates, larynx and trachea. Bronchopneumonia may be present, usually confined to the anteroventral areas, and is characterized by consolidation and atelectasis.

Histopathology: PPR virus causes epithelial necrosis of the mucosa of the alimentary and respiratory tracts marked by the presence of eosinophilic intracytoplasmic and intranuclear inclusion bodies. Multinucleated giant cells (syncytia) can be observed in all affected epithelia as well as in the lymph nodes (Brown *et al*., 1991). In the spleen, tonsil and lymph nodes, the virus causes necrosis of lymphocytes evidenced by pyknotic nuclei and karyorrhexis (Rowland *et al*., 1971). Brown and others (1991) using immunohistochemical methods detected viral antigen in both cytoplasm and nuclei of tracheal, bronchial and bronchio-epithelial cell, type II pneumocytes, syncytial cells and alveolar macrophages.

Small intestines are congested with lining haemorrhages and some erosions. Large intestines (caecum, colon and rectum) have small red haemorrhages along the folds of the lining, joining together as time passes and becoming darker, even green/black in stale carcasses.

1.8. Immunity:

The surface glycoproteins hemagglutinin (H) and fusion protein (F) of morbilliviruses are highly immunogenic and confer protective immunity. PPRV is antigenically closely related to rinderpest virus (RPV) and antibodies against PPRV are both cross-neutralizing and cross protective (Taylor, 1979a). A vaccinia virus double recombinant expressing H and F glycoproteins of RPV has been shown to protect goats against PPR disease (Jones *et al*., 1993) though the animals developed virus-neutralizing antibodies only against RPV and not against PPRV. Capripox recombinants expressing the H protein or the F protein of RPV or the F protein of PPRV conferred protection against PPR disease in goats, but without production of PPRV-neutralizing antibodies (Romero *et al*., 1995) or PPRV antibodies detectable by ELISA (Berhe *et al*, 2003). These results suggested that cell-mediated immune responses could play a crucial role in protection. Goats immunized with a recombinant baculovirus expressing the H glycoprotein generated both humoral and cell-mediated immune responses (Sinnathamby *et al*., 2001). The responses generated against PPRV-H protein in the experimental goats are also RPV cross-reactive suggesting that the H protein presented by the baculovirus recombinant 'resembles' the native protein present on PPRV (Sinnathamby *et al*., 2001). Lymphoproliferative responses were demonstrated in these animals against PPRV-H and RPV-H antigens (Sinnathamby *et al*., 2001). N-terminal T cell determinant and a C-terminal domain harboring potential T cell determinant(s) in goats was mapped (Sinnathamby *et al*., 2001). Though the sub-set of T cells (CD4+ and CD8+ T cells) in PBMC that responded to the recombinant protein fragments and the synthetic peptide could not be determined, this could potentially be a CD4+ helper T cell epitope, which has been shown to harbor an immunodominant H restricted epitope in mice (Sinnathamby *et al*., 2001).

Identification of B- and T-cell epitopes on the protective antigens of PPRV would open up avenues to design novel epitope based vaccines against PPR.

Sheep and goats are unlikely to be infected more than once in their economic life (Taylor, 1984). Lambs or kids receiving colostrum from previously exposed or vaccinated with RP tissue culture vaccine were found to acquire a high level of maternal antibodies that persist for 3-4 months. The maternal antibodies were detectable up to 4 months using virus neutralization test compared to 3 month with competitive ELISA (Libeau *et al*., 1992). Measles vaccine did not protect against PPR, but a degree of cross protection existed between PPR and canine distemper (Gibbs *et al*., 1979).

Though PPR disease can be effectively controlled by RPV vaccine, rinderpest eradication programmes have been launched in many countries and if these campaigns are successful, *Office International des Epizooties* (OIE) recommends the cessation of vaccination of all the animals with RPV vaccine so that any residual foci of RPV could be identified. Under these circumstances, small ruminants could only be protected against PPR by using homologous attenuated vaccine. In addition, the successful use of an attenuated PPRV vaccine against RPV has been reported in goats, opening the possibility to use it as a differentiable vaccine for cattle (Couacy-Hymann *et al*., 1995).

1.9. Diagnosis

Goats and sheep can be infected with RP and PPR as well. Clinical differential diagnosis is not possible as similar disease is produced by both viruses in small ruminants. Therefore, tentative clinical diagnosis may have to be confirmed by laboratory analysis. Diagnosis of PPR may be performed by virus isolation, detection of viral antigens, and nucleic acid sequencing and detection of specific antibody in serum.

1.9.1. Virus isolation

Samples for virus isolation include heparinized blood, eye and nasal swabs (from live animals), tonsil, mesenteric lymph nodes, spleen, section of colon and lung. For successful isolation, samples must be collected during the hyperthermic phase (Lefevre, 1987) and submitted to the testing laboratory in cold ice. The most widely used cell culture systems are primary lamb kidney and ovine skin (Gilbert and Monnier, 1962; Laurent, 1968, Taylor and Abegune, 1979) and Vero cells (Hamdy *et al*., 1976).

The sensitivity of virus isolation technique could be increased when the virus is grown in lamb and goats kidney cells (Taylor, 1984). Vero cells are however widely used for their continuity and low liability of contamination. PPRV has also been adapted to grow in other continuous cell lines including MDBK and BHK-21 (Lefèvre, 1987). Vero cells, derived from African green monkey kidney are currently the most widely used cell line for PPRV and RPV. A culture of Vero cells from American type cell culture (ATCC # CCL81) was found to yield very high titres and is currently used in many laboratories working on PPRV and RPV. Appearance of cytopathic effects (CPE) may require at least 8-10 days or several blind passages. In Vero cells, the cytopathic effects (CPE) produced by PPRV consist of cell rounding, clumping into typical grape-like clusters, formation of small syncytia and appearance of long fine often anastomosing "spindle cells" (Hamdy *et al*., 1976). Like other morbilliviruses, PPRV produces eosinophilic intracytoplasmic and intranuclear inclusion bodies both in primary cells (Laurent, 1968) and continuous cell lines (Hamdy *et al*., 1976).

T-lymphoblast cell line transformed by *Theileria parva* proved to be more sensitive when compared to other cell culture and gave a result within 24 hours (at least 6 days for other cell culture) for both PPRV and RPV (Rossiter, 1994).

Once isolated in cell culture, a candidate PPRV may be identified by one of the three procedures:

- animal inoculation: PPR causes clinical disease in goats and sheep but not in cattle (Gibbs *et al*., 1979);

- reciprocal cross neutralization (differential neutralization): PPRV is neutralized by both PPR and RPV reference sera, but is neutralized at greater titre with the homologous serum (Taylor and Abegunde and, 1979, Taylor, 1979a);
- molecular techniques: cDNA probe, (Diallo *et al*., 1989a, Pandey *et al*., 1992), electrophoretic profile in polyacrylamide gel (PAGE) (Diallo *et al*., 1987) and PCR, (Barret *et al*., 1993, Forsyth *et al*., 1995, Couacy-Hymann *et al*., 2002).

1.9.2. Antigen detecting methods:

1.9.2.1. Agar Gel Immunodiffusion Test

Agar gel immunodiffusion test (AGID) is widely used and can detect 42.6% of antemortem specimens and necropsy specimens (Obi, 1984; Abraham and Berhan, 2001). It can be used to test the presence of both antigen and antibodies and can give results within 2-4 hours when RP hyperimmune serum is used while it needs 4-6 hours with PPR hyperimmune serum (Obi, 1984). One of the important advantages of this test that it is highly specific (92%), though it can not differentiate between PPR and RP.

1.9.2.2. Hyperimmune serum:

Standard antiserum is made by immunising sheep with 5 ml of PPR virus with a titre of 10^4 TCID50 (50% tissue culture infective dose) per ml given at weekly intervals for 4 weeks. The animals are bled 5-7 days after the last injection. Standard RP hyperimmune antiserum is also effective in detecting PPR antigen.

1.9.2.3. Counter immunoelectrophoresis

Counter immunoelectrophoresis (CIEP) is in the same principle as the AGID except that the gel is electrically charged to improve the sensitivity of the test.

1.9.2.4. ELISA for antigen detection:

A monoclonal antibody-based sandwich ELISA was found to be highly sensitive in detection of antigen in tissues and secretions of infected goats (Saliki *et al*., 1994). Another format of antigen

ELISA which is more widely used is immunocapture ELISA (Fig. 1-11). It utilizes MAb directed against the nucleocapsid protein (Libeau *et al*., 1994). It can give a reliable result within two hours in precoated plates and from samples maintained at room temperature for a period of seven days with no more than 50% reduction in response (Libeau *et al*., 1994). The immunocapture ELISA allows a rapid differential diagnosis of PPR or rinderpest viruses, and this is of great importance as the two diseases have a similar geographical distribution and may affect the same animal species. The detecting MAbs used in immunocapture ELISA are directed against two non overlapping domain of the N-protein of PPR and RP, but the capture antibody detects an epitope common to both RP and PPR (Libeau *et al*., 1994). The test is very specific and sensitive, it can detect $10^{0.6}$ $TCID_{50}$/well for the PPR virus and $10^{2.2}$ $TCID_{50}$ for the rinderpest virus. This discrepancy between the two viruses in the assay may be due to a difference in the affinity of the detection antibody for the different N proteins. The main advantages of this assay are:

- Rapidity, it can be performed in a precoated plate in less than 2 hours;
- Specificity ;
- Robustness, it can be carried out on samples which have not been kept under ideal conditions and where no viable virus is present;
- Simplicity.

The immunocapture ELISA is suitable for routine diagnosis of rinderpest and PPR from field samples such as ocular and nasal swabs.

1.9.2.5. cDNA probes:

For the differentiation between PPR and RP, the use of [P^{32}]-labelled cDNA probes derived from the N-protein gene of the two viruses had been described (Diallo *et al*., 1989a). It could differentiate between the two viruses without need for virus isolation. cDNA directed against the matrix protein, fusion protein and phosphoprotein gene were found to cross hybridise to a much greater extent and were not suitable for use as discriminating probes (Diallo *et al*.,1989a).

Unfortunately, this hybridization cannot be used widely because it requires fresh specimens and in addition to the short half life of [P^{32}], there is constraints with the handling of isotopes. Therefore, probes using non radioactive labels such as biotin (Pandey *et al*., 1992) or dioxin (Diallo *et al*., 1995) were developed. The biotin labeled cDNA was found to be as specific as the one using the radioactive label and more rapid in differentiation between PPR and RP (Pandey *et al*., 1992). However, it was reported elsewhere, that the expected sensitivity had never been obtained using non-radioactive labels (Diallo *et al*., 1995).

1.9.2.6. Reverse transcription polymerase chain reaction (RT-PCR)

Conventional serological techniques and virus isolation are normally used to diagnose morbillivirus infection in samples submitted for laboratory diagnosis. However, such techniques are not suitable for use on decomposed tissue samples, the polymerase chain reaction (PCR), has proved invaluable for analysis of such poorly preserved field samples.

Saiki and others (1988) first demonstrated the efficiency of amplifying *in vitro* a selected sequence flanked by two oligonucleotide primers of opposite orientation. The method consists of repetitive cycles of DNA denaturation, primer annealing and extension by a DNA polymerase effectively doubling the target with each cycle leading, theoretically, to an exponential rise in DNA product. The replacement of the polymerase Klenow fragment by thermostable polymerase derived from *Thermus aquaticus* (Taq) has greatly improved the usefulness of PCR. Using this system, a rate of amplification up to 10^7 to 10^9 times has been reported. The efficiency achieved actually can vary enormously, however, since it is dependent on factors such as the number of cycles, the quantity of the starting material, the length of the target DNA, the temperature conditions of annealing and priming, and the polymerase used. When the starting material is DNA, high purification of the nucleic acid is not necessary so the procedure is greatly simplified. These qualities have made the PCR one of the essential techniques in molecular biology today and it is starting to have a wide use in laboratory disease diagnosis.

Since the genome of all morbilliviruses consists of a single strand of RNA, it must be first copied into DNA, using reverse transcriptase, in a two-step reaction known as reverse transcription/polymerase chain reaction (RT-PCR). RT-PCR has been shown to be useful for the rapid detection of morbillivirus-specific RNA in samples submitted for laboratory diagnosis (Shaila *et al.*, 1996). It has proved especially useful in identifying the new morbilliviruses found in marine mammals (Barrett *et al*., 1993b). Both genus-specific and universal morbillivirus primer sets have been produced that can be used to distinguish all known morbilliviruses (Forsyth and Barrett, 1995).

Two sets of primers have been made, based on sequences in the 3' end of N genes (messenger sense), which are least conserved regions between the two viruses. They enable specific amplification of 300 base pair (bp) fragments for RPV and PPRV (Couacy-Hymann *et al*., 2002). Reverse transcription-polymerase chain reaction tests (RT-PCR) using phosphoprotein (P) universal primer and fusion (F) protein gene specific primer sets to detect and differentiate between PPR and RP were described (Barrett *et al*., 1993b; Forsyth and Barret, 1995; Couacy-Hymann *et al*., 2002).

1.9.3. Serology:

Many tests have been used for the demonstration of PPR antibodies in serum: virus neutralization test, agar gel diffusion test, immunoelectrophoresis and recently blocking and competitive ELISA.

1.9.3.1. Virus neutralisation:

The virus neutralisation test (VNT) is sensitive and specific, but time-consuming and expensive. The standard neutralisation test is carried out in roller-tube cultures of primary lamb kidney cells or Vero cells when primary cells are not available. VNT is the most reliable test for detection of morbillivirus antibodies (Rossitter, 1994). Serum against either PPR or RP may neutralise both

viruses, but would neutralize the homologous virus at a higher titre than the heterologous virus. Therefore for differentiation purpose reciprocal cross neutralization is used (Taylor and Abegunde, 1979).

1.9.3.2. cELISA

Competitive and blocking ELISA based on monoclonal antibodies specific for N-protein (Libeau *et al*., 1995) and H-protein (Anderson and Mckay, 1994; Saliki *et al*., 1993; Singh et al., 2004) were developed for detection of antibodies in animal sera. These tests either used gradient purified virus or expressed antigens. In the N-protein cELISA, the serum antibodies and the MAb compete on specific epitope on nucleoprotein obtained from recombinant baculovirus. Though no cross reaction in N-protein cELISA was reported, a high level of competition up to 45% was observed among the negative (Libeau *et al*., 1995). Despite the fact that neutralizing antibodies are not directed against the N-protein, but the H-protein (Diallo *et al*., 1995), a correlation of 0.94 between VNT and cELISA was observed suggesting that the former was more sensitive (Libeau *et al*., 1995). The relative sensitivity of this cELISA to VNT was 94.5, while the specificity was 99.4%. Both blocking ELISA and cELISA detecting anti-H antibodies are based on competition between an anti-H monoclonal antibody (MAb) and serum antibodies, but in case of blocking ELISA the test sera are preincubated with antigen and then incubated with the MAb (Saliki *et al*., 1993). The sensitivity and specificity of the H-blocking ELISA were found to be 90.4% and 98.9% respectively (Saliki *et al*., 1993). PPR cELISA using MAb directed against the H-protein cross reacted to some extent with rinderpest, while RP cELISA is specific, therefore an animal was assumed to have experienced RP if it is positive in both PPR and RP ELISA (Anderson and McKay, 1994). The protocol of cELISA is illustrated in Fig. 1-13. The absorbance in PPR ELISA is converted to percentage of inhibition (PI) using the formula:

PI=100-(absorbance of the test wells/ absorbance of the mAb control wells) x 100. Sera showing PI greater than 50% are scored positive. The overall specificity of c-ELISA test was 98.4% with a

sensitivity of 92.2% when compared with VNT. The diagnostic efficacy of the assay in terms of sensitivity and specificity was calculated using two-sided contingency table (Singh *et al*., 2004). Sensitivity of the assay was taken as proportion of positive samples out of actual positive samples. Specificity was calculated as proportion of negative samples out of total negative samples. The anti-H RP cELISA has been successfully used for serological monitoring of post vaccination herd immunity in the Pan African Rinderpest Campaign (PARC) project to control and eradicate rinderpest from the African continent and which later became part of the FAO Global Rinderpest Eradication Project (GREP). Its wide spread use in (OIE) rinderpest serological surveillance run into controversial difficulties as apparent missing of antibody detection in rinderpest lineage II.

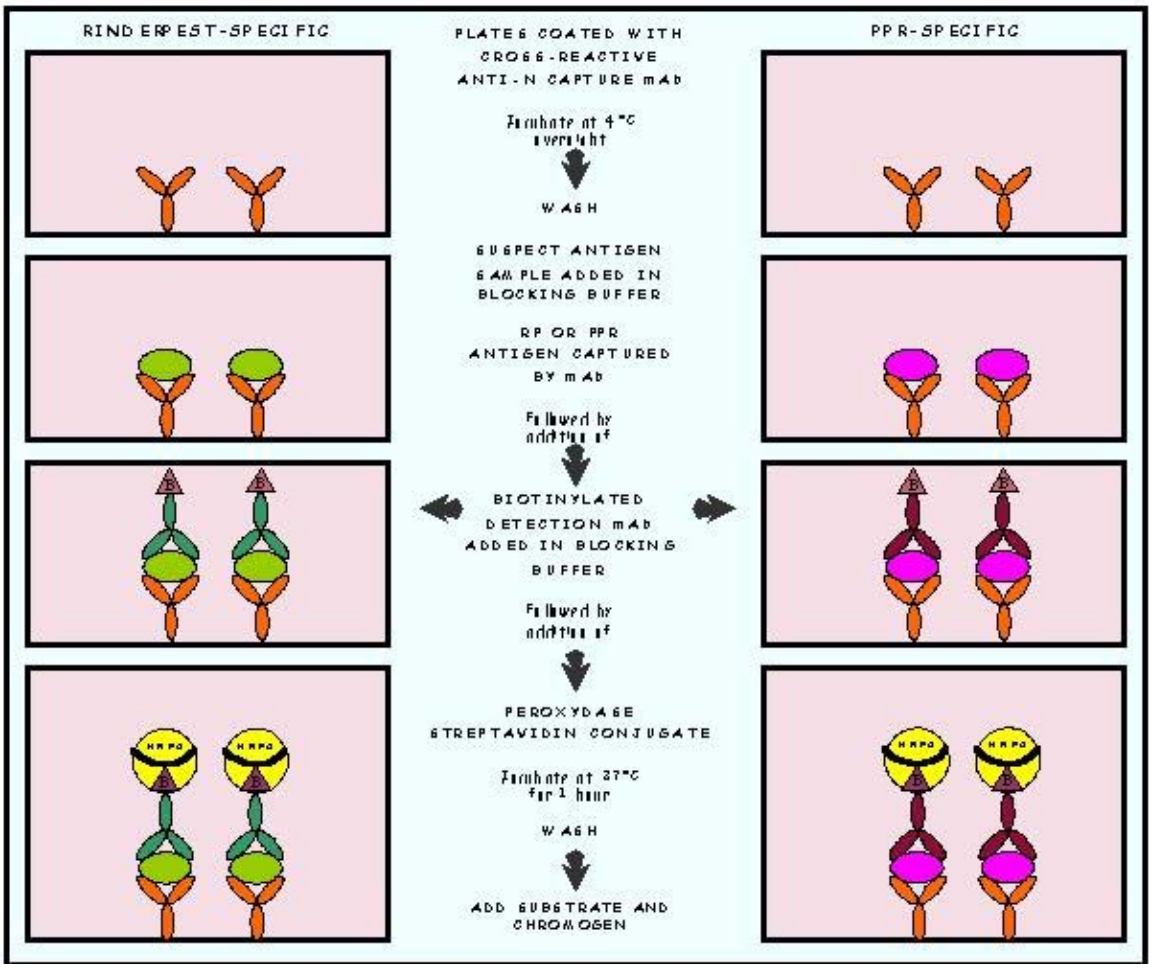

Fig. 1-11 Immunocapture ELISA (Image, Dr G. Libeau)

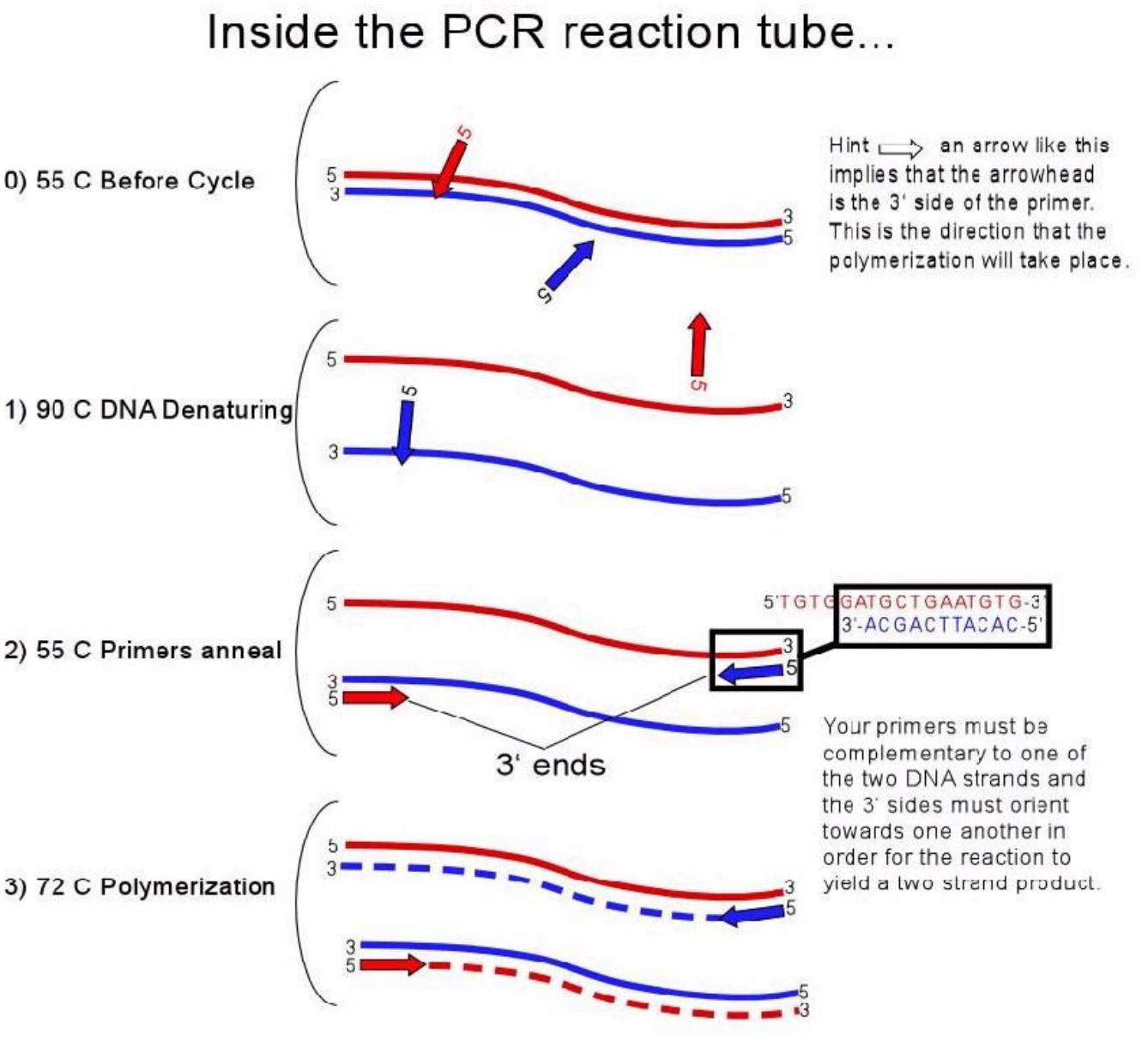

Fig. 1-12 PCR reaction steps (Image, anonym, unpublished)

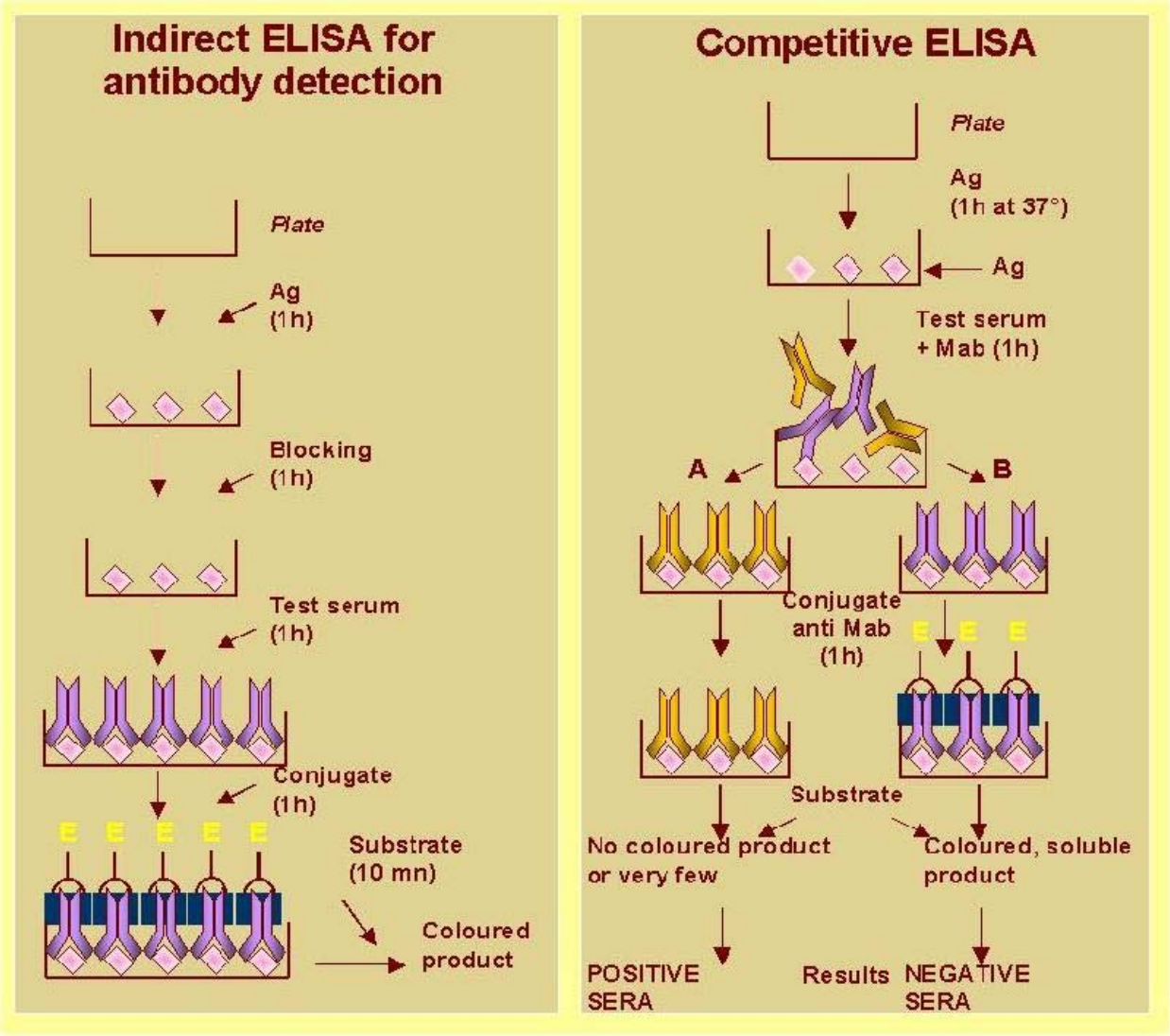

Fig. 1-13. Indirect and Competitive ELISA for antibody detection (Dr G. Libeau)

1.10.Control and prophylaxis:

There is no specific treatment against the disease. Control of PPR in non infected countries may be achieved using classical measures such as restriction of importation of sheep and goats from affected areas, quarantine, slaughter and proper disposal of carcasses and contact fomites and decontamination of affected premises in case of introduction. Control of PPR outbreaks can also rely on movement control (quarantine) combined with the use of focused ("ring") vaccination and prophylactic immunization in high-risk populations. Immunization of small ruminants with lymph node and spleen materials containing virulent virus inactivated with 1.5-5% chloroform was tried and the animals were immune to subsequent challenge 18 months latter (Braide, 1981). Until recently, the most practical vaccination against PPR was based on the use of tissue culture adapted rinderpest vaccine. Vaccination of animals with RP attenuated virus has been practiced for a long time. The tissue culture rinderpest vaccine (TCRV) at a dose of $10^{2.5}$ $TCID_{50}$ protected goats against PPR for 12 months and the animals were not able to transmit the infection following challenge with PPR virus (Taylor, 1979a), although the antigen was detected in lachrymal swabs from vaccinated animals after challenge with virulent virus (Gibbs *et al*., 1979). However, it was reported previously that considerable residues of virulence were detected after 32, 42, even 65 serial passages in embryonic lamb kidney cells (Taylor, 1979a). This vaccine was successfully used to control PPR in some countries in west Africa (Bourdin, 1973) and is widely used in many African countries (Lefèvre and Diallo, 1990). It has been withheld from being used because of its interference with the Pan-African Rinderpest Campaign (PARC), since it is impossible to determine if seropositive small ruminants have been vaccinated or naturally infected with RPV. Sera from animals vaccinated with RP vaccine contain substantial level of RP antibodies with little or no cross neutralising antibodies to PPR but after challenge with PPR, neutralizing antibodies to PPR increase sharply. RP thermostable vaccine was developed for

protection of goats against PPR (Stem, 1993). Homologous PPR vaccine attenuated after 63 passages in vero cell (Diallo *et al*., 1989b) was used and produced a solid immunity for 3 years (Diallo *et al*., 1995). The PPRV homologous vaccine was found to be safe under field conditions even for pregnant animals and it induced immunity in 98% of the vaccinated animals (Diallo et al., 1995). The PPRV vaccine has been tried for protection of cattle against RP and it was found very effective (Couacy-Hymann *et al*., 1995). PPR vaccine seed is available through the Pan African Veterinary Vaccine Centre (PANVAC) at Debre Zeit, Ethiopia, for Africa, or CIRAD-EMVT at Montpellier, France, for other areas.

1.11. Disease Economy:

The PPR epidemics can cause mortality rates of 50–80% in naive sheep and goats populations (Kitching, 1988). Due to the confusion with other diseases, the economic impacts of PPR are probably underestimated, but it is believed that PPR is one of the major constraints of small ruminant farming in the tropic (Taylor, 1984). Based on assumption that goats experience an outbreak every 5 years, Opasina and Putt (1985) estimated an annual sum ranging from 2.47£ per goat at high loss and 0.36 £ per goat at lowest. The loss due to PPR in Nigeria was estimated to be 1.5 million dollars annually (Hamdy *et al*., 1976). The economic losses due to PPR alone in India have been estimated annually to 1,800 millions Indian Rupees (39 millions US$) (Bandyopadhyay, 2002). An economic analysis for assessing benefits of vaccination against PPR in Niger revealed that such a programme was highly beneficial with an anticipated net present value (NPV) return in five years of 24 millions USD following an investment of two millions USD.

Chapter 2

Comparative biology of PPRV among other morbilliviruses

2.1 Introduction

Peste des petits ruminants virus (PPRV) is a very recent addition to the *Morbillivirus* genus in comparison to measles virus (MV), canine distemper virus (CDV) and rinderpest virus (RPV). The disease PPR was described as a clinical entity only in 1942 (Gargadennec and Lalanne, 1942). Measles, canine distemper and rinderpest have been known to exist for several centuries (Appel *et al*., 1981). PPRV was isolated in cell culture (Gilbert and Monnier, 1962) at least 10 years after the three other morbilliviruses were cultured in the early 1950s. For a long time after the description of PPR and even after the agent was isolated, it was thought to be a variant of RP that was adapted to goats and sheep and had lost its virulence for cattle (Laurent, 1968).

PPR is an important disease in its own right, but it has also created problems because of its apparent similarity to rinderpest. The clinical signs of rinderpest in small ruminants closely resemble those of PPR, making differential diagnosis difficult. It should, however, be kept in mind that clinical disease caused by rinderpest in small ruminants is a relatively rare event, even in Asia.

2.2 Pathogenicity and host range

Since morbilliviruses do not persist in an infectious form following an acute infection, and infection results in life-long immunity in recovered hosts, the virus relies on a constant supply of new susceptible hosts for its maintenance. It has been estimated that a population of at least 300,000 individuals is required to maintain MV in circulation (Black, 1991). Each of the morbilliviruses has its different natural host ranges (Appel *et al*., 1981). MV causes disease only in primates. Non-human primates are highly susceptible to MV, but their numbers are too small to maintain the virus in circulation and infection occurs through contact with humans. Canine

distemper virus is naturally pathogenic in the Canidae (dog, wolf, fox), Mustilidae (ferret, mink, weasel), Tayassuidae (javelins) (Appel *et al*., 1991) and Procyonidae (raccoon) families. The recent outbreaks of distemper in seals in Lake Baikal (Visser *et al*., 1993), in lions in the Serengeti National Park (Roelke-Parker *et al*., 1996), and in leopards and other large cats in zoos (Appel *et al*., 1994) have underscored the ability of CDV to invade new host species. Phocine distemper virus, the most recently described member of the *Morbillivirus* genus can infect many species of seal (Duignan *et al*., 1995; Osterhaus, 1992). Little is known of the host range of the cetacean morbillivirus, but serological evidence for its presence in many species of cetacean has been obtained (Duignan *et al*., 1995). Rinderpest virus is primarily a pathogen of cattle and water buffaloes but also could cause disease in goats, sheep, and rarely in pigs (Murphy *et al*., 1999) and most species belonging to other Artiodactyla. Dogs fed infected meat can develop antibodies to RPV, indicating a subclinical infection (Rossiter, 1994). Peste des petits ruminants virus occurs in goats and sheep but it has been described in captive wild small ruminants belonging to three families: Gazellinae (dorcas gazelle), Caprinae (Nubian ibex) and Hippotraginae (gemsbok) (Furley *et al*., 1987).

Another difference among morbilliviruses is the ability of some members to establish persistent infections in their natural hosts. Acute MV infection occurs mainly in childhood and is characterized by fever, cough, coryza, conjunctivitis and skin rash. In about $1/ 10^6$ cases, a fatal degenerative neurological condition known as subacute sclerosing panencephalitis (SSPE) may occur 4-10 years after the acute clinical disease of measles (Appel *et al*., 1981). A related condition designated measles inclusion body encephalitis (MIBE) has also been described (Norrby and Oxman, 1990). Similarly, a very rare but fatal progressive motor and mental deteriorating condition known as old dog encephalitis (ODE) has been described in dogs many years after acute CDV infection (Appel *et al*., 1981). No carrier state or persistent infections have been described for the RPV, PPR or CDV.

Cellular receptors and tropism: Cellular receptors are one of the major determinants of the host range and tissue tropism of a virus. Human signaling lymphocyte activation molecule (SLAM; also known as CD150), a membrane glycoprotein expressed on some lymphocytes and dendritic cells (Cocks *et al*., 1995), is a cellular receptor for MV (Tatsuo *et al*., 2000). The tissue distribution of human SLAM can explain the pathology of measles. Selective infection and destruction of SLAM positive cells may be the principal mechanism underlining the immunosuppressive effect of morbilliviruses in general (Tatsuo *et al*., 2000). The target cells for RPV are epithelial cells, activated lymphocytes, and macrophages (Rey Nores *et al*., 1995). Field isolates of CDV also replicate in dog or ferret macrophages (Brugger *et al*. 1992) as well as in primary dog brain cell cultures (Zurbriggen *et al*., 1987). Cell lines such as Vero (African green monkey kidney) which use mainly CD46 receptors do not allow the propagation of field isolates, whereas cell culture adapted CDV strains such as the Onderstepoort vaccine strain are able to replicate in many cell lines (Appel and Gillespie, 1972). It is known that virulence for the natural host may be lost when CDV is adapted to cell culture (Harrison *et al*., 1968). Furthermore, the marmoset B cell line B95a, which is commonly used to isolate MV from clinical specimens (Kobune *et al*., 1990) expresses a high level of SLAM on the cell surface (Tatsuo *et al*., 2000). B95a cells have been shown to be very sensitive to CDV and RPV (Kai *et al*., 1993, Kobune *et al*., 1991). Vero, 293T (human kidney), and L (mouse fibroblast) cells developed syncytia after transfection with the H and F genes of the RPV Kabete O strain. Thus, SLAMs appeared to act most efficiently as receptors for MV, CDV, and RPV, respectively (Castro *et al*., 1999).

Morbilliviruses use SLAMs of their respective host species as cellular receptors (Tatsuo *et al*., 2001). However, MV, CDV, and RPV strains could use SLAMs of non host species as receptors, albeit at reduced efficiencies (Tatsuo *et al*., 2001). Thus, the finding that these three morbilliviruses use SLAMs as cellular receptors suggests that the usage of SLAM as a receptor has been maintained from the ancestral virus, accounting for an essential part of the pathogenesis

of morbillivirus infections. Recently, B95a was commonly used to isolate morbilliviruses from clinical specimens (Kai *et al*., 1993). A high level of SLAM expression on B95a cells (Tatsuo *et al*., 2000) appears to be a reason for its usefulness. B95a has been shown to be very sensitive to both virulent field virus and vaccine strains of RPV (Lund and Barrett, 2000). Despite sequence differences, the structure required for the interaction with morbillivirus H proteins may be well conserved among SLAMs of many different species. Therefore, the use of SLAM as a cellular receptor may be included in their characteristic properties (Tatsuo *et al*., 2001).

2.3. Serologic relationships

A possible relationship between CDV and RPV was first suspected from the observation that dogs fed on rinderpest infected meat became immune to CDV (Polding and Simpson, 1957). For a long time after PPR was first described, and even the virus was isolated (Gilbert and Monnier, 1962), the causal virus was believed to be a variant of RPV partly because sera against the two agents cross-neutralized and vaccination with RPV vaccine protected against PPR (Bourdin, 1973). Initial studies on the relationship among the morbilliviruses were done using classical serological tests (agar gel precipitation, complement fixation, hemagglutination and virus neutralization) and cross protection studies (Orvell and Norrby, 1974).

Cross neutralization has been adopted as a means for differentiating between PPRV and RPV whose host ranges overlap in small ruminants, serum raised against one virus neutralizing the homologous virus at a higher titre than the heterologous one (Gibbs *et al*., 1979). A practical consequence of serologic cross reactivity between morbilliviruses is that diagnostic tests based on polyclonal antibody with notable exception of VNT, are incapable of distinguishing between PPRV and RPV.

2.4 Cross protection studies

Several investigators have examined one sided and or reciprocal cross protection between various morbillivirus pairs: MV/CDV (Appel *et al*., 1984), MV/RPV (Provost *et al*., 1971), RPV/PPRV

(Gibbs *et al*., 1979), and CDV/PDV (Osterhaus *et al*., 1990). Although the results from various studies may differ because of differences in virus strains, type of vaccine, size of inoculum and experimental methods, it can be stated that any two morbilliviruses show some degree of cross protection. The tissue culture rinderpest vaccine TCRV (Plowright and Ferris, 1962) has been shown to provide complete protection against PPR in goats for at least one year (Taylor, 1979a). TCRV had been used in many countries in control of PPR (Lefèvre and Diallo, 1990). Its use, however, complicated global efforts to eradicate RP.

Indeed, after RP is eradicated in cattle, small ruminants may serve as a reservoir from which RPV could re-emerge. Since there is presently no test for distinguishing between vaccine and wild-type RPV, one cannot determine the origin (vaccinal or natural infection) of RPV antibodies in goats and sheep. A homologous attenuated PPR vaccine has been developed (Diallo *et al*., 1989b), but the problem of differentiating between vaccine and wild-type PPRV may still be posed. This may be the stimulating force for developing marked vaccines for both rinderpest and PPR.

2.5 Antigenic relationships

Panels of monoclonal antibodies (MAbs) have been generated to study antigenic relationships among the morbilliviruses (Harder *et al*., 1991, Libeau and Lefèvre, 1990). Two studies (Norrby *et al*., 1985, Sheshberadaran *et al*, 1986) used anti–CDV and anti-MV MAbs to study the antigenic relationships among CDV, MV and RP by virus neutralization and or direct immunofluorescence. It could be concluded:

- Most MAbs specific for the nucleoprotein (N) and fusion (F) proteins reacted with all three viruses, CDV, RPV or MV;
- MAbs to the phosphoprotein (P) and matrix (M) proteins showed only partial cross reactivity with the greatest variation occurring between CDV and MV;
- The H protein MAbs cross reacted at a low level and only between RPV and MV.

Based on the systematic observation of epitopes shared between RPV and either CDV or MV, never between MV and CDV alone, it has been proposed that RPV may be the archevirus of the *Morbillivirus* genus from which the other viruses evolved, CDV having branched off earlier than MV (Norrby *et al*., 1985). Genetic evidence tends to support this proposal. Further more studies with RPV MAbs though more limited in scope, also indicate that, more MAbs cross react between the MV/RPV pair than the CDV/RPV pair (Libeau and Lefèvre, 1990; Sugiyama *et al*., 1991). The two studies that have included PPRV or anti-PPRV MAbs concluded that PPRV was more closely related to RPV than to MV (McCullough *et al*., 1986). However, the results of Libeau and Lefèvre (1990) using anti-RPV MAbs indicate that PPRV may be also as distant from RPV as it is from MV.

Monoclonal antibodies have also been instrumental in the characterization of morbilliviruses recently isolated from wild aquatic mammals: phocine distemper, dolphin morbillivirus (DMV) and porpoise morbillivirus. The available data suggest that:

- Phocine distemper virus (PDV) isolated from seals in Lake Baikal in Siberia is closely related to CDV to be considered a strain of that virus (Visser *et al*., 1990).
- Phocine distemper virus isolated from seals in the North Sea, while being a distinct morbillivirus, is more closely related antigenically to CDV (Harder *et al*., 1991).
- DMV and PMV isolated respectively from dolphin and porpoises in the Mediterranean Sea, are similar but distinct morbilliviruses, more closely related to PPRV and RPV than to CDV or MV (Visser *et al*., 1993).

2.6. The Comparison of proteins of Morbilliviruses

Eight distinct proteins have been described in morbilliviruses (Tables 2-1, 2-2, 2-3). Properties and biological functions of these proteins include three viral structural proteins (N,P and L) which are internal polypeptides complexed with the viral genome to form the nucleocapsid, while the other three (M,F,H) form the virus envelope (Norrby and

Oxmann, 1990). Comparison using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) techniques of the structural proteins for MV (Cattaneo *et al*., 1989), CDV (Orvell, 1980), RPV and PPRV (Diallo *et al*., 1987) detected variability in electrophoretic mobility. Variability in mobility has been detected in the N (Campbell *et al*., 1980), P, M, and H proteins (Rima, 1983; Saito *et al*., 1992). The N, M, F and L proteins appeared to be the most conserved proteins (Table 2-1). The M, F and P proteins of the vaccine strain of PPRV are most closely related to those of DMV (Diallo *et al*., 1994; Haffar *et al*., 1999; Meyer and Diallo, 1995).

Nucleocapsid (N) Protein: The N protein is phosphorylated in at least some strains of MV (Campbell *et al*., 1980), CDV (Campbell *et al*., 1980) and RPV (Grubman *et al*., 1988). It is very susceptible to non specific intracellular proteolysis resulting in the appearance of extra bands on SDS-PAGE gels (Rima, 1983). Its breakdown may be prevented by addition of protease inhibitors. Apart from its physical role of protecting the viral RNA genome, the N protein has been shown to play a role in the immune response to MV. Vaccinia virus (VV) recombinants expressing the N protein (VV-N) of MV have been shown to induce partial protection in mice (Wild *et al*., 1992) and full protection in rats (Brinckmann *et al*., 1991) against MV challenge. Since N protein immunized individuals do not produce any neutralizing antibody, the mechanism of protection is most likely a cell mediated response. CD4+ T-cell response has recently been obtained using lymphocytes from MV N proteins immunized mice (Giraudon *et al*., 1991). The N protein of MV may play a critical role in viral assembly since it has been shown that vaccinia virus (VV) expressing the N gene of MV could assemble into nucleocapsid like structures (Spehner *et al*., 1991).

Mobility differences have been detected in the N and M proteins of RPV (Anderson *et al*., 1990; Diallo *et al*., 1987) and the N protein of PPRV (Taylor *et al*., 1990). In RPV

the N protein from strains of low virulence migrate faster than the N protein from more virulent strains (Diallo *et al*., 1987). This difference, however, is not useable as a virulence marker since the highly attenuated RBOK vaccine strain has an N protein of intermediate mobility similar to its virulent ancestor, the RBOK wild-type. In PPRV, all isolates from the African continent have N protein that migrates at a slightly faster rate than the N proteins of isolates from the Arabian Peninsula (Taylor *et al*., 1990). This difference has been proposed as a biochemical marker for differentiation of the two groups of PPRV isolates (Lefèvre and Diallo, 1990).

Phosphoprotein: The polymerase associated (P) protein is a minor component of virions. It is phosphorylated (hence the acronym phosphoprotein) in at least some strains of all morbilliviruses (Diallo *et al*., 1987). Phosphorylation may be partly responsible for observed variations in the electrophoretic mobility of the P proteins of various CDV and MV strains (Orvell, 1980). Because of its association with the nucleocapsid, the P protein is thought to be required for formation of an active transcription complex (Norrby and Oxman, 1990). A similar role has been shown for the P protein of Newcastle disease virus, another *Paramyxoviridae* (Hamaguchi *et al*., 1983). The P protein of MV may also play a role in the immune response, since VV expressed protein partially protected rats against MV challenge (Brinckmann *et al*., 1991). Finally, the P protein of MV is required for retention of N protein in the cytoplasm, where the latter is needed for encapsidation of replicating genome (Huber *et al*., 1991).

Non structural proteins: The C protein or its putative mRNA has been identified in cells infected by MV (Bellini *et al*., 1985), CDV (Hall *et al*., 1980), RPV (Grubman *et al*., 1988) and PDV (Blixenkrone-Moller *et al*., 1992; Curran and Rima, 1992). It is transcribed independently of the full length P from a second reading frame on the functionally bicistronic P gene. The V protein or its putative mRNA have so far been

described in cells infected by MV (Cattaneo *et al*., 1989; Wardrop and Briedis, 1991) and PDV (Curran and Rima, 1992). The roles of C and V proteins remain unknown (C increased mRNA transcription in vitro and is an interferon antagonist; V has a putative regulatory role on transcription and replication and is also an inhibitor of interferon response).

The Matrix (M) protein is the smallest in size but it is one of the most abundant of the six structural proteins (Rima, 1983). The M protein may play a role in the immune response as indicated by partial protection of rats against MV using a VV expressed protein (Brinckmann *et al*., 1991). In recent years, research on the M proteins of MV has intensified following the findings that SSPE viruses had a non-expressed M proteins (Hall and Choppin, 1981) and that sera from SSPE patients had high levels of antibodies against all MV structural proteins except M protein (Hall and Choppin, 1981). Subsequently, it was shown that differences between wild-type MV and SSPE viruses involved abnormalities in the expression, structure or stability of not only the M protein (Cattaneo *et al*., 1988) but also the F and H proteins (Baczko *et al*., 1986). Such changes are thought to be responsible for the lack of budding viral particles, infectious virus or cell fusion in the presence of detectable nucleocapsids in SSPE infected brain tissue (Ter Meulen and Carter, 1984). Abnormalities in protein structure and function particularly the M protein, may thus be responsible for establishment or maintenance of MV persistence. Indeed it has recently been shown that normal MV M protein is associated with intracellular viral nucleocapsids whereas M protein from an SSPE strain of the same lineage is localized mainly in the cytosol of infected cells (Hirano *et al*., 1992).

The fusion (F) protein mediates fusion between the virus envelope and cell membrane or between the infected cell and adjacent cells, thereby playing a major role in viral penetration and spread within the host (Norrby and Oxman, 1990). The comparison of

the nucleic acid sequences of different morbillivirus fusion (F) protein genes revealed that the 5'-end sequence of the mRNA is specific to each virus. The F protein plays an important role in the immune response. Purified VV- expressed or ISCOM (immune stimulating complexes) incorporated F proteins have been successfully used to protect rabbits and cattle against RPV (Barrett *et al*., 1989). Dogs against CDV (De vries *et al*., 1988) and rats and mice against MV (Wild *et al*., 1992). Although animals immunized with F protein may generate neutralizing antibodies which are protective, other mechanisms such as inhibition of cell fusion may be important in protection, since non-neutralizing MAbs induced protection of mice against lethal CDV challenge (Hirayama *et al*., 1991). Recent sequencing data on SSPE viruses indicate that alterations in the F protein cytoplasmic domain may also play a role in establishment of MV persistence (Schmid *et al*., 1992).

The hemagglutinin (H) protein is responsible for the virus-cell membrane interaction for binding the cellular receptors. Among morbilliviruses, only MV has an H protein with hemagglutinating properties, although sera against CDV and RPV inhibit hemagglutination by MV (Orvell and Norrby, 1974). The H protein may also play a role in cell to cell fusion as indicated by the capacity of anti-H MAbs to inhibit cell to cell fusion in vitro (Wild *et al*., 1991). The role of the H protein in protection has been extensively investigated. Vaccinia virus-expressed or ISCOM incorporated H proteins induce complete protection against RPV in cattle (Giavedoni *et al*., 1991) and CDV in mice (Hirayama *et al*., 1991). Virus neutralization is an important protection mechanism, since passively administered anti-H neutralizing MAbs induce full protection in mice (Varsanyi *et al*., 1987). Inhibition of cell to cell fusion may also be a protection mechanism. Furthermore, MAbs against the H proteins of MV have been shown to decrease the expression of the P, F, and M proteins a phenomenon called (antibody-

induced antigenic modulation) which may also play a role in protection (Fujinami *et al*., 1984).

The polymerase (L) protein is the largest polypeptide of morbilliviruses (Rima,1983). The L protein is a very minor viral component, its gene being the last to be transcribed. In association with the N and P proteins, it forms the nucleocapsid. The L protein is also necessary to the transcription or ribonucleoprotein complex (Hamaguchi *et al*., 1983). Because of its large size the L protein is believed to exhibit the major RNA dependent RNA polymerase activities (nucleotide polymerization, capping and polyadenylation) of viral mRNA (Kingsbury, 1990).

2.7 Genetic relationships

2.7.1. Nucleotide and amino acid sequence homologies

Full length genome sequences are available for MV (Cattaneo *et al*., 1989), RPV (Baron and Barrett, 1995), CDV (Barrett *et al*., 1987), PPRV (Bailey *et al*., 2005) and the dolphin morbillivirus (DMV) (Rima *et al*., 2003). The MV-RPV and CDV-PDV pairs exhibit the closest relationship. The nucleotides or amino acid sequence may be a good indicator of the degree of relatedness. A graphical representation of the similarity between PPRV and other members of the *Morbillivirus* genus showed regions of high and low nucleotide conservation. Regions of high conservation include the L and M genes. Non-coding regions show very little similarity (Bailey *et al*., 2005). The PPRV genome encoded the same eight proteins as the type virus (MV), also its length was divisible by six, a feature shared with other *Paramyxoviridae* (Calain and Roux, 1993). The genome was most similar at the nucleotide (nt) level to that of RPV. The full length genome sequence shows that the N, V and H proteins of PPRV had close similarity with DMV, indicating a close antigenic relationship between the two viruses (Bailey *et al*., 2005).

Table 2-1 Comparative electrophoretic mobilities of Morbillivirus proteins

	Estimated molecular weight				Postanslational modification
	CDV	MV	RP	PPRV	
Polymerase (L)	180-200	180-200	200-212	200	Glycosylated
Hemagglutinin (H)	76-85	79-80	74-81	78	Phosphorylated
Phosphoprotein (P)	66-73	70-72	71-92	82-86	Phosphorylated
Nucleocapsid (N)	58-60	59-60	65-68	60-62	Glycosylated
Fusion (F_0)	59-62	59-62	62	-	
F_1 subunit	40-41	40-41	49.5	-	
F_2 subunit	16-23	16-23	-	-	Glycosylated
Matrix (M)	34-35	34-35	38	39	
Non structural (C)	15	18-20	19	19.5	
Non structural (V)	-	40	-	-	

Molecular weights are expressed in kilodaltons (Kd). (Diallo et 1987)

Table 2-2 Homology at amino acid sequence level in percentage

Virus pair	N	P	M	F	H
MV-CDV	67	45	76	66	37
MV-RPV	74	-	78	77	60
MV-PDV	69	45	77	57	38
CDV-RPV	-	-	77	66	38
CDV-PDV	76	76	90	83	74
RPV-PDV	-	-	73	56	37

Table 2-3 Protein homology of morbilliviruses

Protein compared	CDV	MV	RPV	PPRV
N	77	64	66	69
P	75	43	47	na
M	91	78	76	77
F	83	57	56	68
H	75	33	32	36
L	90	72	73	na

Barrett, 1999 *na = data not available

2.8. Phylogenetic relationships

On the basis of phylogenetic analysis of morbilliviruses, it is thought that when cattle were domesticated, they passed a morbillivirus, a progenitor of modern RPV, to humans, which eventually evolved into MV. Similarly, carnivores could have contracted a morbillivirus infection from their ruminant prey, which then evolved into CDV (Barrett and Rossiter, 1999). MV and RPV are closely related, and CDV and phocine distemper virus are the most distantly related to MV and RPV among morbilliviruses (Barret and Rossiter, 1999). Furthermore, among all viral proteins, the H protein is the least conserved among CDV, RPV, and MV (37% identity between CDV and MV) (Blixenkrone-Moller, 1993). PDV antibodies, were found in sera obtained from Arctic seals (Barrett, 1999). Arctic seals may have been infected with CDV by contact with terrestrial carnivores that can carry the virus (wolves, foxes, dogs, polar bears) and have evolved into the phocid virus (Barrett, 1999).

2.9. Conclusions

PPRV exhibits the typical characteristics of the *Morbillivirus* genus in the *Paramyxoviridae* family. PPRV is not only a distinct virus but may be less closely related to RPV than MV to RPV. Data from the three members of the *Morbillivirus* genus (MV, CDV, and RPV) indicate that strains of varying pathogenicity may occur naturally. In RPV for example strains of low virulence have been identified (e.g. RBT/1 and Reedbuck). Such strains distinguish themselves from virulent strains by having a faster migrating N protein (Diallo *et al*., 1987) or by their MAb reactivity spectrum (Libeau and Lefèvre, 1990). The available PPR isolates have been the subject of only a few studies and there is no indication that they differ in pathogenicity. Variations in clinical disease of PPR in Africa may have resulted from breed resistance or a default in appropriate cellular receptors rather than of variations in virulence of the PPRV. Therefore, the

epidemiology, pathogenicity and host resistance of PPR is more complex than thought earlier. It is hoped that marked RP and PPR vaccines and diagnostic tests capable of differentiating infected and vaccinated animals will improve the diagnostic and epidemiosurveillance capability. Control of PPR using tissue culture PPR homologous vaccine is currently underway in endemic countries. However, the predominant husbandry system in Africa where most households keep a few free ranging goats or sheep, means that vaccination coverage of the population will be difficult to attain in small ruminants.

Scope of the thesis

The ability of PPRV to infect and produce disease (pathogenicity) in a range of animals appears to be variable based on breed and species of animals, endemic situation and eco-climatic (environmental) or field conditions. The hypothesis was that infection with PPRV does not always result in clinical disease. Field serosurveillance was conducted to determine the probability of infection in several species of animals in Ethiopia. From the observation that the virus could circulate without inducing clinical signs in some susceptible species, it was decided to carry out *in vitro* studies on the virulence of PPRV. Virulence of PPRV was first monitored by measuring the severity of infectivity in different cell culture systems. Three cell lines, which are frequently used for morbillivirus replication *in vitro*, were infected with a defined MOI of virus and monitored for a period of time sequence. Infectivity of PPRV was compared with RPV using virus specific monoclonal antibodies.

The infectivity of the virus varied and indicated differences in cell permissivenes and cellular tropism. Cellular receptors are one of the major determinants of the host range and tissue tropism of a virus. However, virus and cellular interaction is a complex process involving attachment and fusion genes, cellular environment and immune status of animals. This approach was completed by a molecular analysis of the H protein, the virus ligand of the cell receptor and of the virus promoters, comparing strains of different virulence. The objective was to identify molecular determinants for virulence.

OBJECTIVES

The objectives of the study were to:

1. Undertake studies on epidemiology of PPR in Ethiopia: In Africa, goats are severely affected while sheep undergo a mild form or rarely suffer clinical disease. PPR is one of

the most important economical diseases in Ethiopia. Clinical PPR is confirmed in Ethiopian goats, however, its circulation in other animals has never been described. The apparent absence of pathogenicity in these animals may have been due to host resistance or loss of virulence of the virus strain. The objective of the present work is to detect the antibody seroprevalence in camel, cattle, goat and sheep, which may confirm natural transmission in these animals without clinical disease.

2. Monitor PPR virulence in cell culture and analyse the H protein: PPRV exhibited different levels of virulence between sheep and goats. Goats were severely affected while sheep generally underwent a mild form. However, the virus circulation in both species was the same level, suggesting a difference in host susceptibility. In addition, other species, the camel, was infected by the virus but without showing clear disease. This difference may result from a difference in cell susceptibility to the virus. The cell susceptibility can be affected by the rate of infection, the affinity of the virus for its cell membrane receptor, the attachement (H) protein, the efficiency of intercellular spreading of the virus and capacity to induce damages in infected cells. However, the factors which determine the virulence remain largely unknown and have not been related to a single event. Low virulence outcome may result from a lesser infection of the cells or a lower replication of the virus in the cells, both resulting in a lower viral antigen distribution through different organs and tissues. Such characteristics may account for the milder clinical disease and lower mortality. The capability of cells to be infected and support active virus replication has important implications on the pathogenesis of the disease. The second objective was designed to monitor sensitivity of different cell lines to PPRV.
3. Sequence analysis of genome and antigenome promoters of wild-type and vaccine strains of PPRV: molecular basis for attenuation of the virus vaccine strain was sought with comparative sequence analysis of the genome and antigenome promoters (GP and AGP).

As demonstrated by Banyard *et al*. (2005) in the case of RPV, the virus genome promoters play a role in the pathogenicity of morbilliviruses. Thus, GP and AGP of the peste des petits ruminants (PPR) virus, amplified from different pathological samples of sheep and goat origins, were sequenced and compared with corresponding sequences of PPR vaccine strain and other morbilliviruses.

Chapter 3

PPR occurrence in Ethiopia

3.1. Introduction

Ethiopia, is 471,776 sq miles (1,221,900 sq km) which is located in the Horn of Africa (Fig. 3-1). It borders with Eritrea in the north, Djibouti in the northeast, Somalia in the east and southeast, Kenya in the south, and the Sudan in the west. The capital city is Addis Ababa, which is located in the center of the country.

3.1.1 Population

The population of Ethiopia in 2004 is estimated at 71.066 million comprising 59.867 million rural (84 percent) and 11.199 million urban (16 percent), respectively. The male female ratio is approximately 50%. The overall annual population growth rate is estimated at 2.8 percent.

3.1.2. The Agricultural Sector

Ethiopia is comprised of several major ecological zones leading to extreme variations in agro-climatic conditions and diverse genetic resource bases. Agriculture is the cornerstone of the economy, where small-holder farmers are responsible for 96% of the cropped area. The crops grown varied according with soil types ranging from vertisol to sand, and cropping altitudes ranging from less than 600 m to more than 3,000 m above sea level. The main cereal staples are wheat, barley, teff (*Eragrostis abyssinica*), finger millet, maize and sorghum. Cash crops include coffee, oilseeds and spices.

Rainfall has two distinct seasons: the *belg*, a minor season that usually begins in January – February and ends in April–May and the *meher* or *kiremt*, the main rainy season, which starts in June–July and ends in September–October.

3.1. 3. The Livestock Sub-Sector

Livestock production is an integral part of the country's agricultural system and is determined by ecology, climate, and it’s economic importance for the farmer. Common grasslands provide extensive pasture and browse in Afar, Somali, the southern zones of Bale, Borena and South Omo, and in the western lowlands that reach from Gambella to Tigray.

Livestock are significant components of small scale and pastoralist farming systems and are reserves for family emergency needs. Manure is the cheapest and easily available fertilizer to increase soil fertility. Draft oxen are used for ploughing the land for crop production. Sheep and goats supply more than 30% of the domestic meat consumption. Animals and their products including hides and skins are major export commodity, which is estimated to be 50 million USD per annum. Pastoralists and semi-pastoralists sustain their culture, life style and pride on their livestock.

The livestock population is estimated at 35 million tropical livestock units (TLU) which includes 30 million cattle, 21 million sheep, 24 million goats, 7 million equids, one million camels and over 53 million chicken. The main cattle breeds include the Arsi (highland zebu), Boran, Fogera, Horo, Sheko (Gimira), Abigar (Nuer), and the Adal. The Fogera and Horo, well known for their milk, are reared around lake Tana and Eastern Welega regions, respectively. The Boran, a beef breed, is found in the southern and eastern parts of the country, while the Gimira and Abigar breeds in the south-west are considered to have tolerance to high tse-tse challenge. European breeds, especially Friesian and Jersey, have been imported and used for cross breeding with the indigenous animals.

Sheep and goat breeds include the Horro, Menz, Adal (Afar) Arsi and Black-Head Ogaden Sheep, and the Adal (Afar) goat. Few exotic breeds of sheep and goats have been introduced for cross breeding. Awassi and Corriedale sheep have been used in the highlands while the Anglo-

Nubians are kept for milk and meat production in the lower altitude of the mixed farming systems.

3.1. 4. Animal Health

Animal diseases have a significant impact on household food security. Some of these diseases wipe out the entire herds and threaten the livelihoods of the farmers. They contribute to a general decline in the productivity and have been determinant factors for poverty in rural communities. Animal diseases affect productivity by 50 to 60% a year by reducing production potentials of the indigenous stock and restricting the introduction of more productive exotic breeds.

The major causes of economic loss and poor productivity in livestock is the prevalences of a wide ranges of diseases (CBPP, FMD, CCPP, PPR, AHS and HS among others) and parasites (Trypanosomiasis, Anaplasmosis, other external and internal parasites). The direct losses due to mortality is estimated 8-10% of cattle, 14-16% of sheep flock and 11-13% of goat per annum. Indirect economic losses occur through slow growth, low fertility and decreased work output. Three major impacts of diseases were: socio-economic 85% (primarily production losses and control costs incurred by the poor), zoonotic (for those diseases transmissible from animals to humans) and national average of 15% a combination of marketing impacts on the poor with public-sector expenditures on disease control. Therefore, impact of animal diseases on the economy is that, they impede investment in the sector, and are sanitary barriers of export markets. Therefore, improving health and productivity would provide an important opportunity for increasing food security and could be a promising and cost effective way of stimulating the national economy.

While recognising the extent of the animal health challenges, it is important to note the success of recent efforts to eradicate rinderpest. Ethiopia has formally declared itself provisionally free of rinderpest to the Office International des Epizooties.

3.2. PPR in Ethiopia

PPR was suspected on clinical grounds to be present in goat herds in Afar region of Eastern Ethiopia in 1977 (Pegram *et al*., 1981). Moreover, serological and clinical evidences were reported by Taylor (1984). However, the presence of the virus was only confirmed in 1991 with cDNA probe in lymph nodes and spleen specimens collected from an outbreak in a holding land near Addis Ababa. PPR was characterized by ocular and nasal discharges, mouth lesions, pneumonia, gastro enteritis and diarrhoea (Figs. 1-8, 1-9). The disease in this outbreak caused more than 60% mortality (Fig. 3-2).). The disease probably was introduced into Ethiopia in 1989 in the Southern Omo river valley from where it moved eastward to Borena region and then northwards along the Rift valley to Awash (Gopilo *et al*., 1991, Roeder *et al*., 1994). The disease became endemic in goats (Abraham and Berhan, 2001).

Small ruminants in this country mainly thrive on free-range pasture land, shrubs and forest cover. Due to the shrinkage in pasture land and forest area, these animals move to long distance in search of fodder and water during dry season. This phenomenon is common due to different summer and winter grazing grounds depending upon the altitude. PPR is transmitted through direct contact between infected animal and susceptible population. During nomadism, animals come in contact with local sheep and goat population from where they pick up the infection or spread disease if nomadic flock is pre-exposed. Therefore, migratory flocks play an important role in transmission epidemiology of PPR. Movement of animals and introduction of newly purchased animals from the market also play an important role in transmission and maintenance of the virus. This could be one of the possible reasons for higher frequency of PPR outbreaks between March to June (Fig. 3-3), which also correspond to lean period of kidding. Although seasonal occurrence of PPR virus outbreaks is disputed, disease transmission is certainly affected by animal movement for which socioeconomic factors and variations in agro climatic conditions are responsible. Large group of animals move to large areas and even between

different districts. With the start of rains, the movement of animals is restricted due to the easy availability of local fodder. Nutritional status of the animals also gets improved during the rains. This may reduce disease transmission after the start of rains and during the period of easy availability of fodder. Similar observations were also recorded in tropical humid zone of Southern Nigeria during a period of 5 years of observations (Taylor, 1984).

Fig 3-1 Map of Ethiopia

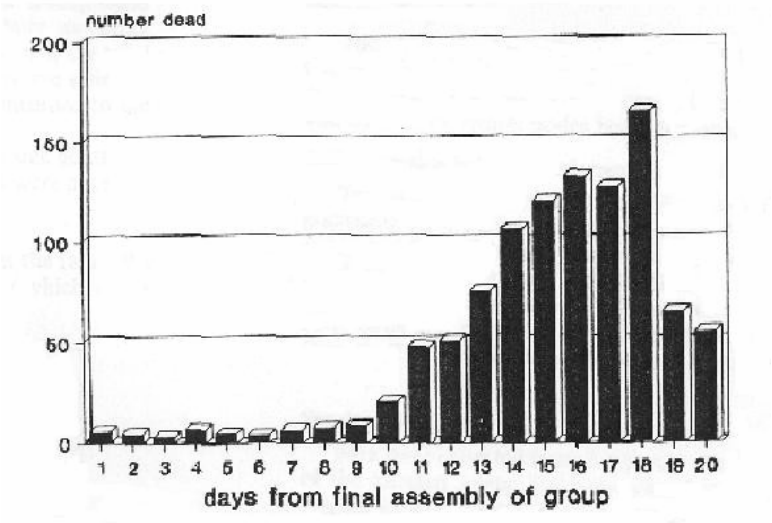

Fig. 3-2 PPR point epidemic in goats in one gathering site for marketing. (Roeder *et al*., 1994)

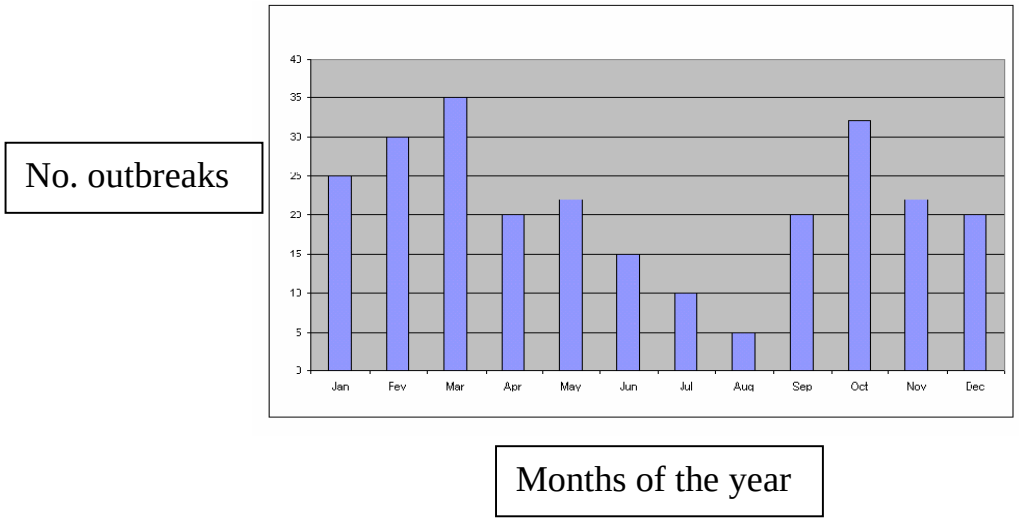

Fig. 3-3 PPR has seasonal disease pattern in endemic areas. (Animal health reports, 2001)

In Ethiopia, PPR in goats was described and confirmed by the laboratory. However, there was always a question if other species of animals (camel, cattle and sheep) could be infected in the absence of detectable clinical signs. Our first work was to address this question. The following published article describes the antibody seroprevalence which was carried out in these animals.

Chapter 3

Article 1

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Antibody seroprevalences against peste des petits ruminants (PPR) virus in camels, cattle, goats and sheep in Ethiopia

G. Abraham [a,b], A. Sintayehu [a], G. Libeau [b,*], E. Albina [b], F. Roger [b], Y. Laekemariam [a], D. Abayneh [a], K.M. Awoke [a]

[a] *National Animal Health Research Centre, P.O. Box 04, Sebeta, Addis Ababa, Ethiopia*
[b] *TA30/G, EMVT, CIRAD, Campus International de Baillarguet, 34398 Montpellier Cedex 5, France*

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Abstract

A questionnaire-survey data indicated that 26% of 276 farmers reported the presence of respiratory disease in their herds in 2001. The incidence was perceived as "high" in small ruminants and camels, but as "low" in cattle. Simultaneously, 2815 serum samples from camels ($n = 628$), cattle ($n = 910$), goats ($n = 442$) and sheep ($n = 835$) were tested. The peste des petits ruminants (PPR) antibody seroprevalence was 3% in camels, 9% in cattle, 9% in goats and 13% in sheep. The highest locality-specific seroprevalences were: camels 10%, cattle 16%, goats 22% and sheep 23%. The animals had not been vaccinated against rinderpest or PPR. Antibody seroprevalences detected in camels, cattle, goats and sheep confirmed natural transmission of PPR virus under field conditions.

Keywords: ELISA; Ethiopia; Peste des petits ruminants; PPR; Rinderpest

1. Introduction

Peste des petits ruminants (PPR) is an acute and highly contagious viral disease of small ruminants, which is characterised by high fever, ocular and nasal discharge, pneumonia, necrosis and ulceration of the mucuous membrane and inflammation of the gastro-

* Corresponding author.
E-mail address: genevieve.libeau@cirad.fr (G. Libeau).

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intestinal tract leading to severe diarrhoea (Gibbs et al., 1979). Goats are affected severely but sheep undergo a mild form of the disease (Lefèvre and Diallo, 1990) and cattle have a sub-clinical infection (Anderson and McKay, 1994). Recently, PPR antigen was detected in an outbreak of respiratory disease in camels (Roger et al., 2000). Morbidity and mortality risks in small ruminants vary but can be as high as 100 and 90%, respectively. These risks are usually lower in endemic areas and mortality can be as low as 20% maintained in the newborns (Lefèvre and Diallo, 1990; Roeder and Obi, 1999) unless complicated with other concurrent infections.

The PPR virus belongs to the genus *Morbillivirus* in the family Paramyxoviridae. Morbilliviruses form a small group of antigenically related viruses: measles virus, rinderpest virus, canine-distemper virus, phocine-distemper virus, and dolphin and porpoise morbilliviruses (Barrett et al., 1993).

PPR first was reported from West Africa in the early 1940s (Gargadennec and Lalanne, 1942) and later recognized as endemic in West and Central Africa (Scott, 1981). It has been reported in the Sudan (El Hag Ali and Taylor, 1984), Kenya and Uganda (Wamwayi et al., 1995) and in Ethiopia (Roeder et al., 1994). It is endemic in the Arabian Peninsula, the Middle East and in the Indian sub-continent (Shaila et al., 1996).

PPR-virus isolates can be grouped into four distinct lineages on the basis of partial sequence analysis of the fusion (F) protein genes; lineage III is the lineage found in Eastern Africa (Ethiopia).

In arid and semi-arid zones, where the endemic form persists, PPR acts as a predisposing factor for secondary bacterial infections (Lefèvre and Diallo, 1990). After the first confirmed cases of PPR in goats in Ethiopia (Roeder et al., 1994), seasonal outbreaks were reported in many parts of the country (Abraham and Berhan, 2001). Serum antibodies have been detected recently in flocks of sheep with respiratory disease complex in central Ethiopia (Tibbo et al., 2001).

Our objective was to determine antibody seroprevalence to PPR of unvaccinated camels, cattle, goats and sheep and to test association with owner-perceived "respiratory disease" complex.

2. Materials and methods

2.1. Survey

The study area was divided into five geographically separate strata within the pastoral production system (Fig. 1). Three hundred villages were selected randomly (using a table of random numbers) from list of villages provided in designated localities. Two hundred and seventy-six villages were visited because the remaining 24 had problems of accessibility. In each village, a randomly (using a table of random numbers) selected owner was interviewed to ask whether any of his animals experienced respiratory disease in the last 9 months, whether it occurred in specific seasons, and whether there had been any death from this disease. At the same time, serum samples were collected from watering points (where most of the animals were gathered). Herds within the same village shared grazing area and watering points, and were considered as single village-level clusters and sampling units (regardless of

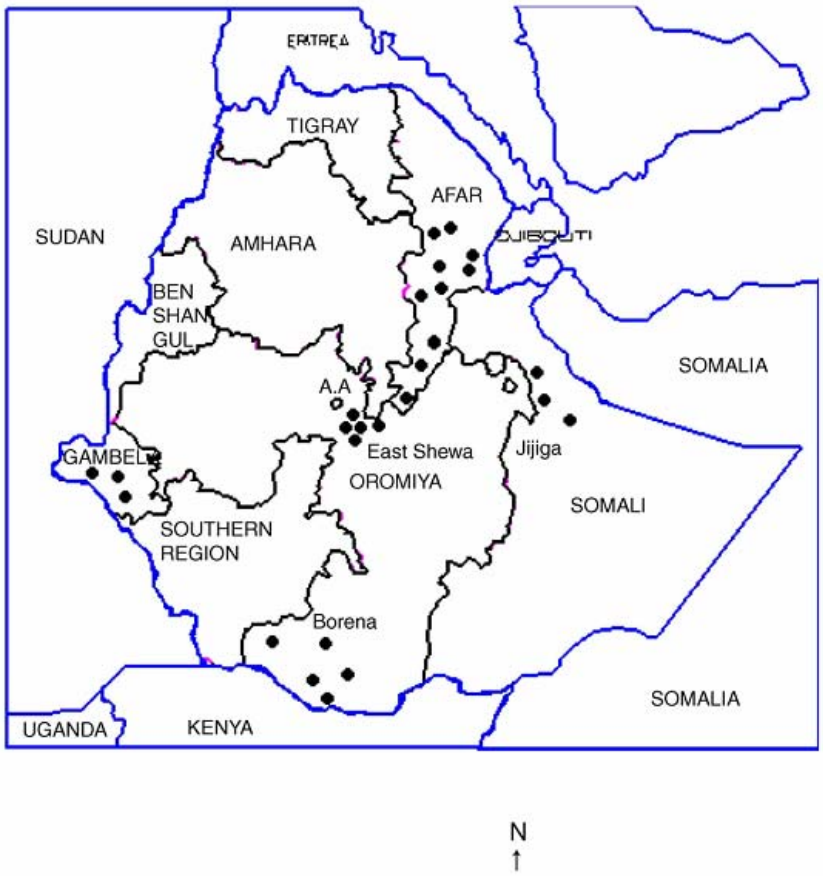

Fig. 1. Map of Ethiopia with indicated sites (●) of "respiratory disease" questionnaire-survey and peste des petits ruminants serosurvey in 2001.

owner or species, all the animals in one village mingle as one massive herd in terms of PPR transmission potential). The number of samples was proportional (1%) to the population estimates of each species of animals in a given village. The 2815 serum samples collected were from 628 camels, 910 cattle, 442 goats and 835 sheep. The age group of sampled animals was in the range of 1–3 years to rule out maternal antibody (>1 year) and to discover recent infection (<3 years). The animals were born after declaration of provisional freedom from rinderpest and cessation of vaccination in these localities. Data and sera were collected and processed within a month and half up to October 2001.

2.2. Serum collection

Blood was collected by jugular-vein puncture using venoject needles and vacutainer tubes (Venoject, UK). The blood was left to clot overnight in cold boxes or small field

refrigerators. Serum was decanted into sterile tubes and kept on ice for transportation to the laboratory. In the laboratory, the serum was centrifuged to remove the remaining red blood cells before being transferred to 2-ml cryovials and stored at −20 °C.

2.3. Competitive enzyme linked immunosorbent assay (C-ELISA)

PPR C-ELISA kit and the assay protocol were supplied by the Institute for Animal Health (Pirbright Laboratory, UK). According to the manufacturers, the antigen was prepared by propagating the PPR virus (Nigerian strain 75-2) in Madin Darby bovine kidney (MDBK) cell culture. The culture was harvested when there was 90% cytopathic effect and the supernatant was concentrated by ammonium-sulphate precipitation. The test is based upon the competition between the anti-H protein monoclonal antibody and antibodies in the serum samples for binding to the antigen. The presence of antibodies in the serum sample will block reactivity of the monoclonal antibody (MAb) resulting in the reduction of expected coloration following the addition of enzyme-labelled anti-mouse conjugate and substrate–chromogen solution. The positive/negative cut-off was established by testing previously known negative sera (by virus-neutralization test) from cattle, goats and sheep. The highest competition value for these sera was 45% (unpublished data). Therefore, 50% competition was adopted as cut-off for routine testing. The manufacturers also recommended a similar cut-off value.

The ELISA micro-plates were read with an Immunoskan (Flow laboratories, UK) reader with an inference filter of 492 nm. The reader was connected to computer loaded with ELISA Data Information (EDI) software (FAO/IAEA, Vienna, Austria), which was used to automate the reading and calculation of percentage of inhibition (PI) values. The OD values were converted to percentage inhibition by using the following formula:

$$\mathrm{PI} = 100 - \left(\frac{\text{OD control or test serum}}{\text{OD monoclonal control}}\right) \times 100.$$

The samples with PI > 50% (cut-off) were considered as positives. The antibody seroprevalence results were apparent prevalence (AP) and true prevalences were calculated (Rogan and Gladen, 1978): true prevalence = (AP + Sp − 1)/(Se + Sp − 1), where Sp is specificity and Se is sensitivity.

3. Results

A fourth of the farmers interviewed indicated the presence of "respiratory disease" in their herds in the last 9 months in 2001. "High" incidences of "respiratory disease" were reported in goats and sheep in the Afar, Borena, Gambela and Jijiga localities. However, the highest locality-specific PPR antibody seroprevalence was 16% in cattle, 22% in goats and 23% in sheep. "High" incidence of "respiratory disease" also was reported in camels in Afar and Jijiga. The antibody seroprevalece was 10% in camels in Afar and PPR antibody was not detected in camels in Jijiga. The farmers reported "average" incidence of "respiratory disease" in East Shewa and the highest antibody seroprevalence was 16% in sheep. Antibody was not detected in goats and sera were not collected from camels in East

Table 1
Questionnaire-survey on "respiratory disease" in Ethiopian livestock (2001)

Locality	Number of		% of farmers reporting "respiratory disease"	Incidence			
	Districts	Farmers interviewed		Camel	Cattle	Goats	Sheep
Afar	10	95	42	High	Low	High	High
Borena	5	66	20	Average	Average	High	High
East Shewa	5	78	32	NA	Average	Average	Average
Gambela	3	22	18	NA	Low	High	Average
Jijiga	3	15	20	High	Average	High	High

Low: occurs rarely; average: occurs more frequently; high: it is a serious problem; NA: no information is available or no camels are reared in the area.

Table 2
Serological results of peste des petits ruminants in Ethiopia (2001)

Locality	Camels		Cattle		Goats		Sheep	
	No. sampled	Positive (%)	No. sampled	Positive (%)	No. sampled	Positive (%)	No. sampled	Positive (%)
Afar	400	10	400	15	NA	NA	396	16
Borena	160	0	200	5	200	0	160	2
East Shewa	NA	NA	110	5	100	0	111	16
Gambela	NA	NA	120	16	42	22	48	23
Jijiga	68	0	80	5	100	15	120	7
Total	628		910		442		835	

NA: serum samples were not collected.

Shewa. The overall seroprevalence was 3% in camels, 9% in cattle, 9% in goats and 13% in sheep (Tables 1 and 2).

4. Discussion

In Ethiopia, goats react more severely to PPR virus exposure compared to sheep and they exhibit striking clinical signs while sheep undergo a mild form of the disease. However, a similar profile of serological status is widely reported (Taylor, 1984). The antibodies in goats (9%) were slightly lower than in sheep (13%) in this study, which may have resulted from the lower number of samples investigated or from the fact that goats were more susceptible and may have died from the disease, whereas sheep may have survived. This is the first report of PPR antibody detection in cattle and camels in Ethiopia. There was no information on the specificity and sensitivity of the PPR C-ELISA in the kit manual. Therefore, estimates by other laboratories that employed similar anti-H protein monoclonal antibody were accepted. The sensitivity of 90% and specificity 98% was reported (Saliki et al., 1993). The sensitivity was raised to 92% and the specificity remained 98% when the cut-off was lowered to 38% (Sing et al., 2004). Limited cross reaction with rinderpest antibodies were reported (Anderson and McKay, 1994). However, rinderpest has

been eradicated from Ethiopia since 1997. The sampled cattle and other animals were born after cessation of rinderpest vaccination and declaration of provisional freedom in these localities. The animals were not vaccinated against rinderpest or PPR. Therefore, the seroprevalences only could have resulted from field infection with PPR virus. The seroprevalence in cattle (9%) seems higher than those reported in West Africa: 1.78 and 4.5% in Mali and Cameroun, respectively (Tounkara et al., 1996, Ngangnou et al., 1996). It perhaps is explained by higher population density and mixed grazing resulting in increased contacts between small ruminants and cattle. Similarly to Ethiopia, seemingly higher PPR antibody seroprevalence was detected in cattle (15.57%) in Turkey (Ozkul et al., 2002).

"Respiratory disease" was reported of " high" occurrence in camels, goats and sheep in four localities. Considering the seroprevalence of PPR antibodies in camels (10%) in Afar, the role of PPR virus in the "respiratory diseases" should not be ignored in that area. PPR virus antigen was detected in an outbreak of respiratory disease in camels (Roger et al., 2000). Serum antibodies have been detected recently in flocks of sheep with respiratory disease complexes in central Ethiopia (Tibbo et al., 2001). However, these respiratory diseases were complex and of various origins.

Antibody seroprevalences detected in camels, cattle, goats and sheep confirmed natural transmission of PPR virus under field conditions. Further studies are, however, needed to determine if it had any role in the reported "respiratory disease" in these animals.

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From this study, we confirmed that PPRV exhibited different levels of virulence between sheep and goats. Goats were severely affected while sheep generally underwent a mild form. However, the virus circulation in both species was the same level, suggesting a difference in host susceptibility. In addition, another species, the camel, was infected by the virus but without showing clear disease. This difference may result from a difference in cell susceptibility to the virus. The cell susceptibility can be affected by the rate of infection, the affinity of the virus for its cell membrane receptor, the efficiency of intercellular spreading of the virus and capacity to induce damages in infected cells. However, the factors which determine the virulence remain largely unknown and have not been related to a single event. Low virulence outcome may result from a lesser infection of the cells or a lower replication of the virus in the cells, both resulting in a lower viral antigen distribution through different organs and tissues. Such characteristics may account for the milder clinical disease and lower mortality. The capability of cells to be infected and support active virus replication has important implications on the pathogenesis of the disease. Rinderpest virus is primarily a pathogen of cattle and water buffaloes but also could cause disease in goats, sheep, and rarely in pigs (Murphy *et al*., 1999) and most species belonging to other Artiodactyla. Dogs fed infected meat can develop antibodies to RPV, indicating a subclinical infection (Rossiter, 1994). Morbilliviruses use SLAMs of their respective host species as cellular receptors (Tatsuo *et al*., 2001). However, MV, CDV, and RPV strains could use SLAMs of nonhost species as receptors, albeit at reduced efficiencies (Tatsuo *et al*., 2001). Thus, the finding that these three morbilliviruses use SLAMs as cellular receptors suggests that the usage of SLAM as a receptor has been maintained from the ancestral virus, accounting for an essential part of the pathogenesis of morbillivirus infections. Genetically, it was assumed that when cattle were domesticated, they passed a morbillivirus, a progenitor of modern RPV, to humans, which eventually evolved into MV. Similarly, carnivores could have contracted a morbillivirus infection from their ruminant prey, which then evolved into CDV (Barrett and Rossiter, 1999). PPRV has

also probably arisen from a RPV ancestor through adaptation to small ruminants. Interestingly, adaptation of RPV to another species seems to have resulted in a reduction of the host range (basically primates for MV, carnivores for CDV and small ruminants for PPRV). In this context, we were interested to check if PPRV may have different cell affinity than the one already described for other morbilliviruses. The second article was about comparing PPRV and rinderpest (RPV) vis-à-vis three different cell lines.

Chapter 4

Article 2. **Submitted to *Virus Research***

Differences in sensitivity of B95a, MOCL-5, 293T and Vero cells to peste des petits ruminants virus (PPRV)

*G. Abraham, G. Libeau, C. Minet, C. Grillet, Catherine Cetre-Sossah, and E. Albina**

CIRAD- EMVT, TA30/G, Campus International de Baillarguet, 34398 Montpellier, Cedex 5, France.

Abstract

A panel of cells infected with peste des petits ruminants virus (PPRV) and rinderpest virus (RPV) and harvested at different times post infection, were stained with virus specific monoclonal antibodies and with specific mouse antisera (IgG) conjugated with fluorescein isothiocyanate. After fixation they were analysed using flow cytometry and the results were later compared with the cell culture titration. Vero and 293T cell lines supported virus replication of PPRV and RPV. However, antigen was not detected in B95a cells infected with PPRV. Further efforts to adapt PPRV in B95a cells by six blind passages failed to result in productive infection. Differences in sensitivity of B95a cells between PPRV and RPV may reflect differences in the interaction of viral protein with the SLAM (CD150) receptor. Comparative amino-acid sequence analysis of the H proteins of PPRV, RPV, measles virus (MV) and canine distemper virus (CDV) showed conserved critical residues for the interaction with the SLAM. Three point mutations were detected specific for PPRV and hypothetical 3D structural model of globular head of H protein confirmed these modifications at positions 508, 525 and 526. Whether these modifications may account for a defective interaction of the PPRV with B95a cells will need further analysis.

Key words: B95a, H protein, peste des petits ruminants, rinderpest, SLAM, receptor

* Corresponding author: Dr Emmanuel Albina Tel:(33)467593705 or (33)467593724

Fax : (33) 4 67 59 37 98. Email : emmanuel.albina@cirad.fr

1. Introduction

Peste des petits ruminants (PPR) is an acute and highly contagious viral disease of domestic and wild small ruminants, which is characterised by high fever, ocular and nasal discharge, pneumonia, necrosis and ulceration of the mucuous membrane and inflammation of the gastro-intestinal tract leading to severe diarrhoea and death (Gibbs *et al.,* 1979). PPRV exhibits different levels of virulence between sheep and goats (Lefèvre and Diallo, 1990, Roeder *et al*., 1994). However, the factors which determine the virulence remain unknown and have not been related to a single event (Brown and Torres, 1994; Rey Nores and McCullough, 1996). Low virulence outcome may result from a lower replication of the virus, resulting in a lower viral antigen distribution through different organs and tissues. Such characteristics may account for the milder clinical disease and lower mortality (Wohlsein *et al*., 1995). The capability of cells to be infected and support active virus replication has important implications on the pathogenesis and epidemiology of the disease.

PPR and RP viruses are classified in the genus *Morbillivirus* of the family *Paramyxoviridae*. They are genetically and antigenically very closely related to other viruses in the genus. Morbilliviruses are enveloped viruses with non segmented negative-strand RNA genomes. They have two envelope glycoproteins, the hemagglutinin (H) and fusion (F) proteins, mediating receptor binding and membrane fusion, respectively. The H (attachment) glycoproteins of Morbilliviruses are more divergent (Blixenkrone-Müller *et al*., 1996) and may play a role in host cell specificity. Cellular receptors are one of the major determinants of the host range and tissue tropism of a virus (Baron *et al*., 1996). B95a lymphoblastoid cells are a good host for rinderpest virus, but they did not support replication of PPRV unless the virus was adapted through five or six blind passages (Das *et al*., 2000). This appeared to be in contradiction with results obtained with other morbilliviruses in the same cells. RPV (Kobune *et al*., 1991), MV (Kobune *et al*., 1990) and canine distemper virus (Seki *et al*., 2003) replicated and were isolated successfully in

this cell line. SLAM receptors have been used by CDV, MV and RPV and H gene sequences interacted with this receptor in B95a cell infections.

The present work was designed to monitor sensitivity of different cell lines with PPRV and RPV was used as positive control of the assay.

2. Materials and methods

2.1. Cells and virus: Vero (African green monkey), 293T (transformed human embryo kidney) and B95a (marmoset B cell lines) from American Type Culture Collection and MOCL-5 (sheep monocyte derived cell line) (Olivier *et al.,* 2001), were grown in Eagles minimum essential medium (Gibco, UK), Dulbecco's modified medium with L glutamine (Gibco, UK) and RPMI 1640 (Gibco, UK), respectively and supplemented with 10% fetal bovine serum and 1% Glutamine. All cultures were maintained in 5% CO_2 incubator at 37°C.

2.2. *In vitro* infection: The above cultured cells were infected with vaccine strain of PPR virus (Diallo *et al.,* 1989b) and Rinderpest virus vaccine strain (Pirbright, UK). For negative controls, an inoculum of uninfected cell lysate prepared in the same manner that for the virus was used. After an adsorption period of 30 minutes at 37°C, the cell cultures were incubated and harvested at different times post infection (0 – 144 hours).

2.3. Monoclonal antibody (MAb): PPRV anti-N monoclonal antibody (Libeau *et al.,* 1992) and the PPRV anti-H, RPV (anti-H and N) monoclonal antibodies (Pirbright, UK) were used for immuno labelling. The fluorescein conjugated isotype was anti mouse IgG (H+L) (Sigma, USA).

2.4. Flow cytometry: Non-adherent cells were removed by shaking of culture flasks followed by pipetting to dislodge loosely adherent cells. Adherent cells were removed from the tissue culture flasks by incubation with EDTA/trypsin for 5 minutes at 37°C. After being harvested, cells were washed with washing buffer (0.1% PBS-azide, 5% horse serum and 0.1% saponin (w/v) solution). Immunolabelling was achieved in a two step procedure: (I) incubation with

predetermined optimal concentrations of anti-N or anti-H monoclonals at 4°C for 30 minutes and (II) staining with isotype specific mouse antisera (IgG) conjugated with fluorescein isothiocyanate. Each step was followed by two washings with washing buffer, after which the cells were fixed with 1% PFA.

Fluorescence staining was determined by flow cytometry using a FACScan flow cytometer (Becton Dickinson, USA) and the Lysis II software program (CellQuest, UK). Forward scatter and side-scatter profiles were used to place gates on live cells and excluding cell debris. At least 5,000 live cells were acquired.

2.5. Virus infectivity assay: Cell and supernatant fractions from PPR infected and non-infected cultures were harvested at different times post infection (0 – 144 hours) and stored at –70°C until titration. The virus titre was determined by titration of serial 10 fold dilutions on semi-confluent monolayers of cells in tissue culture micro-plates. The virus titre was calculated by the method of Reed and Muench (1938).

2.6. Adaptation of PPRV to B95a cells : Two strains, the Vero-adapted Nigeria 75/1 vaccine strain and the wild type virulent Nigeria 75/1 strain, were serially passaged to six times. The cytopathic effect was regularly checked over the period of 7-10 days for each passage.

2.7. Comparative analysis of H gene amino-acid sequences: The amino-acid sequences of the hemagglutinin protein of PPRV and other vaccine cell-adapted and wild type strains of *Morbillivirus* were aligned using CLUSTAL W included in the Vector NTI-9 package (Informax Inc., USA). Critical residues for H-MV/SLAM (CD150) interaction, identified so far, were placed on the multiple alignments. Specific amino-acid mutations on the H-PPRV in highly conserved positions for other morbillivirus were also identified.

2.8. Modelling : The sequence of the H globular head of the vaccine strain PPRV (Nig75/1, was compared other morbilliviruses : Accession numbers used are PPRV (vac: **X74443**, wt: **AJ512718** and **NC_006383**), RPV (vac: **Z30697**, wt**: X98291**), MV (vac: AF266286 and

AF266289, wt: **AF266288** and **AB012948**), CDV (vac: **AF305419** and **AF378705**, wt: **AF164967** and **AY466011**).

The sequences were then introduced in a 3D model building and visualization using SwissModel (Peitsch, 2005, Guex et Peitsch, 1997, Schwede et al, 2003) and DeepView version 3.7 (Swiss-PdbViewer) and the H-MV model from Massé *et al* (2004) as molecule template.

3. Results

At times indicated, cells were harvested and stained with anti-N and anti-H monoclonal antibodies specific for PPRV or RPV. After staining and acquisition, data were plotted on gated live cells using a 3D histogram (fluorescence intensity (x-axis), number of cells (y-axis) and incubation time (z-axis). The maximum antigen expression was measured at 120 hours post infection (Fig. 1 and 2). The flow cytometry detected viral N antigen in 293T cells earlier, while in Vero cells the N antigen was detected only after 72 hours post infection. Peak fluorescence on both cell populations was achieved between 120 and 144 hours (Fig. 3). The B95a, 293T and Vero cell lines supported rinderpest virus replication and expression. However, B95a cells did not support replication of PPR virus and so PPR virus antigen was not detected in these cells. Further efforts to adapt PPRV in B95a cells by six blind passages failed to result in productive infection. Antigen was not detected in the sheep MOCL 5 cells infected with both PPRV and RPV (data not shown).

In cell culture titration assay CPE was detected in 293T earlier than in Vero cells. Peak titres were detected at 120 hours post infection (Fig. 4). The titres varied with cell type, but clearly both cells were able to support the growth of PPRV and RPV. Results of cell culture titration and flow cytometry analysis were comparable and fairly confirmed the sensitivity of the test.

H gene sequences of PPRV, RPV, CDV and MV retrieved from gene bank database were aligned and sequences were compared.

Amino-acid sequence multiple alignment of the H-protein of different morbilliviruses is shown on Fig. 5. Highly conserved positions are indicated in red letters and critical residues for SLAM (CD150) interaction are in black boxes, within the 501-555 positions. From this alignment, we were interested to identify unique and conserved mutations in H-PPRV compared to the other morbillivirus. Twenty seven positions were thus detected (amino-acids in blue). Interestingly, one mutation V525 → I525 is on a position identified in H-MV as critical for interaction with the SLAM of B95a cells (Massé *et al*, 2004). However, the non polar, hydrophobic aliphatic valine was mutated for an amino-acid sharing the same properties, thus, suggesting a moderate impact on the protein functions. Two other mutations were located in the region of the H protein that is critical for SLAM interaction: V508 → I508 and F555 → V555. Furthermore, we submitted to SwissModel, the amino-acid sequence of the globular head of the H protein of our vaccine strain Nig75/1. Surface modelling was established using as template, the hypothetical 3D structural model of H-MV. The results are shown in Fig. 6. Overall, the critical sight for SLAM interaction is conserved with a part located on the top of the globular head and the second part on one lateral side. However, two differences could be identified between the H-MV and H-PPRV molecules. First, on top side, the surface of critical residues was slightly modified, may be as a consequence of the mutation V508 → I508. In addition, the critical residue L526 on H-MV was buried in our H-PPRV model and replaced by amino-acids Y540 and I542.

4. Discussion

The objective of the current investigation was to determine if there had been any difference in replication and subsequent antigen detection between PPR and rinderpest viruses with cell type. Comparison of the infections showed that the kinetics of replication were variable. Thus the antigen titre at the earlier time points post infection was lower in Vero compared to 293T cells. These results would suggest that replication in 293T cells was initially more efficient. The reason

may relate to the difference levels of cytoplasmic, particularly lysosomal, activity in the cell types. Nevertheless, these differences did not prevent the virus from eventually spreading to all cells in the culture. Moreover, vaccine strains of both PPR and rinderpest viruses are well adapted to Vero cells.

B95a and MOCL5 cells did not support active PPR virus replication. It may be that these cells lack or are deficient in specific attachment protein sequence or an intercellular host protein necessary for efficient virus replication. This may also reflect the use of a different cell receptor (Wild *et al*., 1991). Cellular receptors are one of the major determinants of the host range and tissue tropism of a virus. The H (attachment) glycoproteins of morbilliviruses are variable (Blixenkrone-Müller *et al*., 1996) and this may play a role in host cell specificity. B95a lymphoblastoid cells are a good host for rinderpest virus, but they did not support replication of PPRV. Further efforts to adapt PPRV in B95a cells by six blind passages failed to result in productive infection, contrary to the reports of Das and others (2000). Differences in sensitivity of B95a cells between PPRV and RPV may reflect the use of a different cell receptor, which is believed to be determinant in the host range and tissue tropism. This appeared to be in contradiction with results obtained with other morbilliviruses in the same cells. RPV (Kobune *et al*., 1991), MV (Kobune *et al*., 1990) and canine distemper virus (Seki *et al*., 2003) replicate and can be isolated successfully in B95a cell line. MV uses CD46 receptor for Vero but SLAM for B95a (Takeuchui *et al*., 2002).

Amino-acid sequence analysis of the H protein of several vaccine and wild type strains of PPRV, RPV, measles virus (MV) and canine distemper virus (CDV) showed that the critical residues identified on the H-MV protein for the interaction with the SLAM (CD150 receptor) are conserved in all morbillivirus H sequences we have analysed and surface modelling using hypothetical 3D structural model of H-MV identified one mutation as critical for interaction with the SLAM of B95a cells (Massé *et al*, 2004). Thus, the hydrophobic non polar aliphatic H-MV

residue V525 was replaced by the hydrophobic non polar aliphatic I525. The effect of this conservative mutation on the H-SLAM interaction is probably limited but requires further investigation. In addition, the SwissModel (Schwede *et al.*, 2003), using the amino-acid sequence of the globular head of the H protein detected two differences between the H-MV and H-PPRV molecules. First, on top side, the surface of critical residues was slightly modified, may be as a consequence of the mutation V508 → I508. Second, the critical residue L526 on H-MV was replaced by amino-acids Y540 and I542. Whether these modifications may account for a defective interaction of the H-PPRV with the B95a SLAM receptor will need further analysis, as the non-susceptibility of B95a cells to PPRV cannot be explained simply by sequence modification of the H protein.

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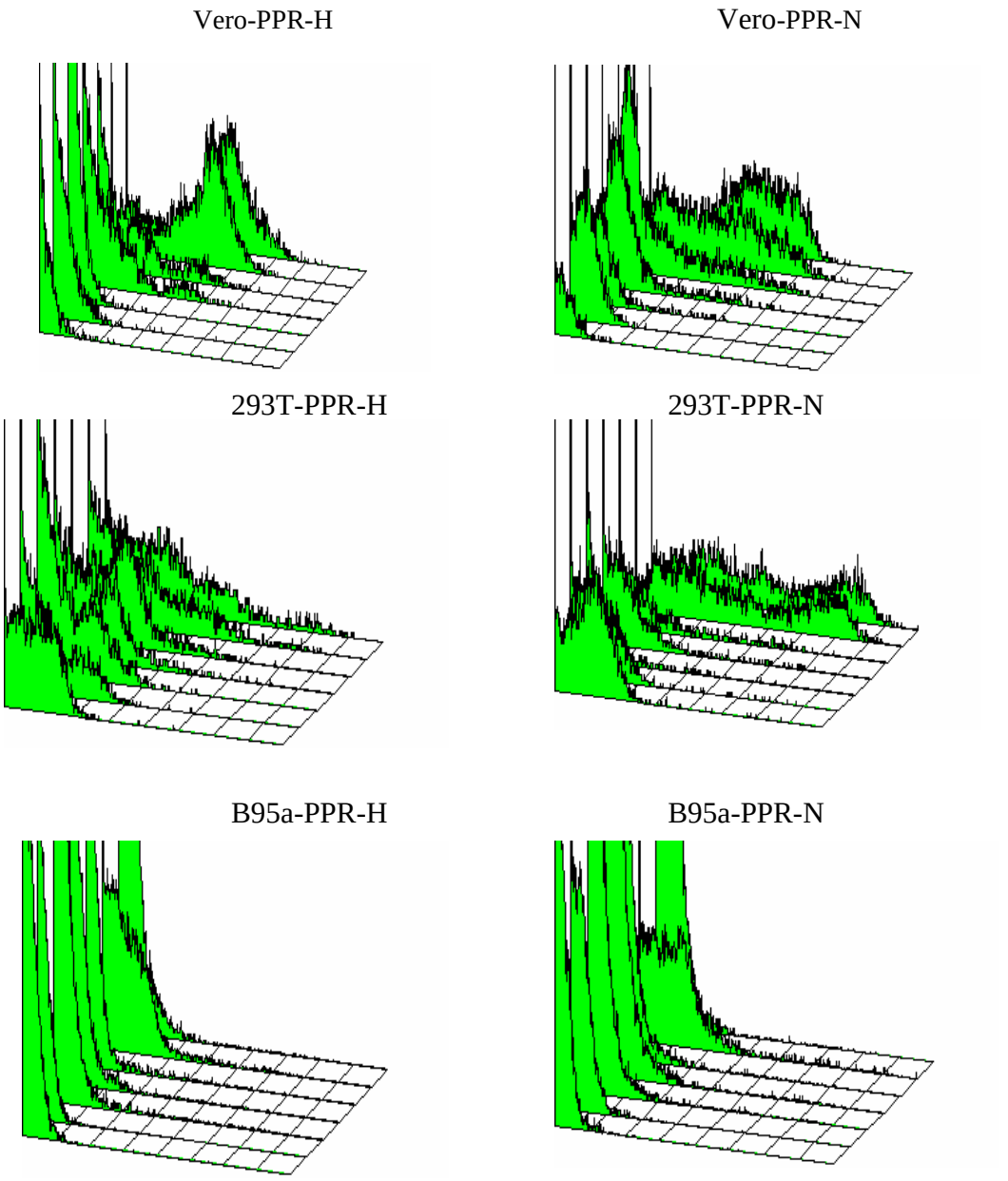


Fig. 1 Flow cytometry analysis of cells infected with peste des petits ruminants virus. The relative cell number (in %) is plotted on the Y-axis(0, 20, 40, 60), fluorescence intensity is plotted on the X-axis (10^1 , 10^2 , 10^3, 10^4 of each square) and time is plotted on the Z – axis (starting from 0, 24, 48, 72, 96, 120, 144 hours post infection for each square).

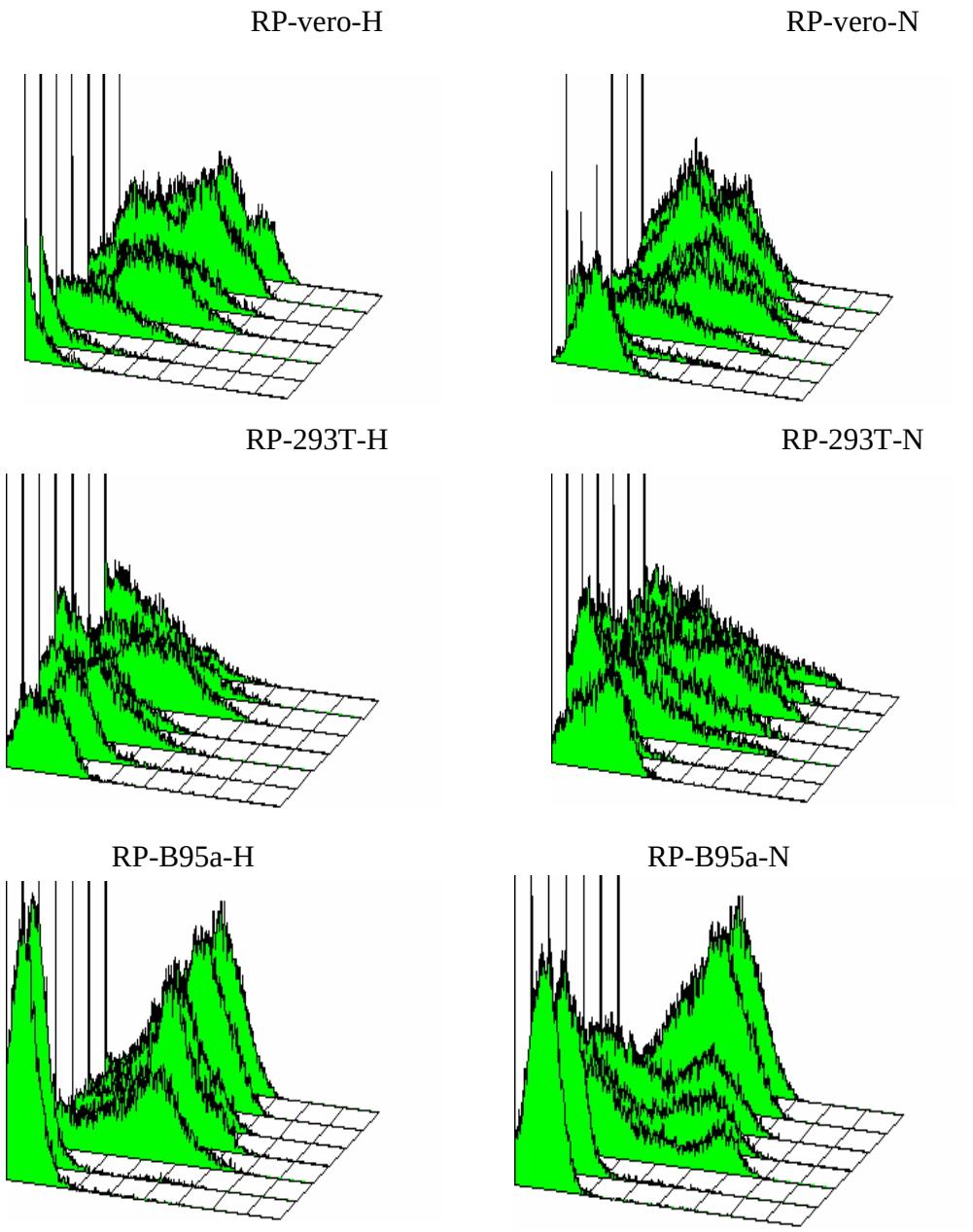
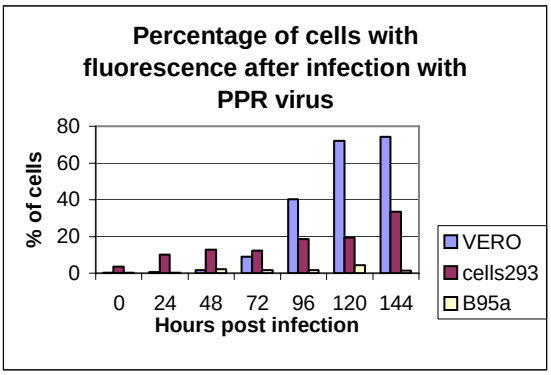


Fig. 2 Flow cytometry analysis of cells infected with rinderpest virus. The relative cell number (in %) is plotted on the Y-axis(0, 20, 40, 60), fluorescence intensity is plotted on the X-axis (10^1 , 10^2 , 10^3, 10^4 of each square) and time is plotted on the Z – axis (starting from 0, 24, 48, 72, 96, 120, 144 hours post infection for each square).



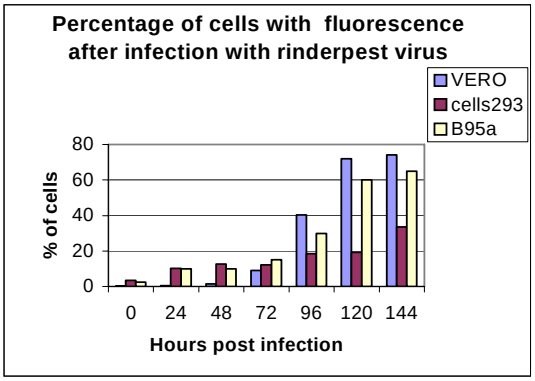
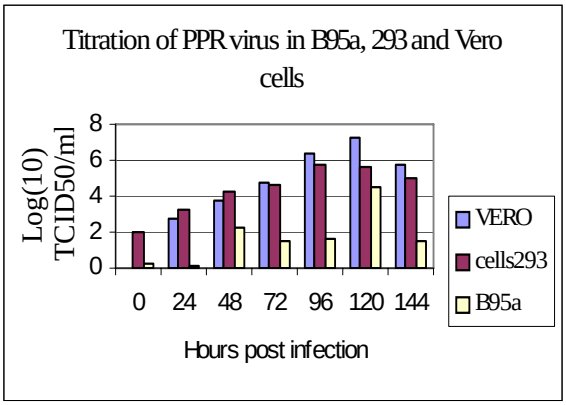


Fig. 3. Percentage of cells indicating positive fluorescence.



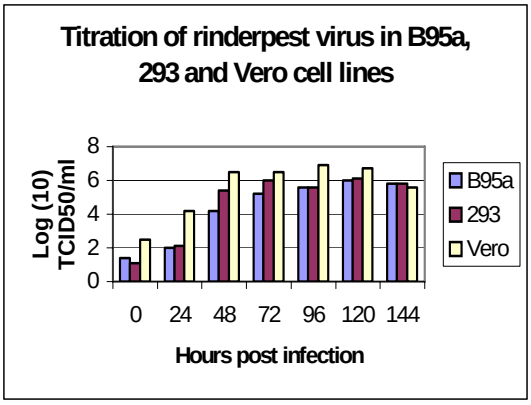


Fig. 4. Titration of Both PPRV and RPV in B95a, 293T and Vero cell lines.

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                    1                                                  50
PPRV (AJ512718)    (1) MSAQRERINAFYKGNPHNKNHRVILDRERLVIERPYILLGVLLVMFLSLI
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PPRV (NC_006383)    (1) MSAQRERINAFYKDNPHNKNHRVILDRERLVIERPYILLGVLLVMFLSLI
   PPRV (X74443)    (1) MSAQRERINAFYKDNLHNKTHRVILDRERLTIERPYILLGVLLVMFLSLI
    RPV (X98291)    (1) MSPPRDRVDAYYKDNFQFKNTRVVLNKEQLLIERPCMLLTVLFVMFLSLV
    RPV (Z30697)    (1) MSPPRDRVDAYYKDNFQFKNTRVVLNKEQLLIERPCMLLTVLFVMFLSLV
   MV (AB012948)    (1) MSPQRDRINAFYKDNPHPKGSRIVINREHLMIDRPYVLLAVLFVMFLSLI
   MV (AF266288)    (1) MSPQRDRINAFYKDNPHPKGSRIVINREHLMIDRPYVLLAVLFVMSLSLI
   MV (AF266286)    (1) MSPQRDRINAFYKDNPHPKGSRIVINREHLMIDRPYVLLAVLFVMFLSLI
   MV (AF266289)    (1) MSPQRDRINAFYKDNPHPKGSRIVINREHLMIDRPYVLLAVLFVMFLSLI
  CDV (AF164967)    (1) MLSYQDKVSAFYKDNARANSSKLSLVTEEQGGRRPPYLLFVLLILLVGIM
  CDV (AY466011)    (1) MLSYQDKVGAFYKDNARANPSKLSLVTEEHGGRRPPYLLFVLLILLVGIL
  CDV (AF378705)    (1) MLSYQDKVGAFYKDNARANSTKLSLVTEEHGGRRPPYLLFVLLILLVGIL
  CDV (AF305419)    (1) MLPYQDKVGAFYKDNARANSTKLSLVTEGHGGRRPPYLLFVLLILLVGIL

                        51                                                100
 PPRV (AJ512718)   (51) GLLAIAGIRLHRATVGTLEIQSRLNTNIELTESIDHQTKDVLTPLFKIIG
PPRV (NC_006383)   (51) GLLAIAGIRLHRATVGTSEIQSRLNTNIELTESIDHQTKDVLTPLFKIIG
   PPRV (X74443)   (51) GLLAIAGIRLHRATVGTAEIQSRLNTNIELTESIDHQTKDVLTPLFKIIG
    RPV (X98291)   (51) GLLAIAGIRLHRAAVNTAKINNDLTTSIDITKSIEYQVKDVLTPLFKIIG
    RPV (Z30697)   (51) GLLAIAGIRLHRAAVNTAKINNDLTTSIDITKSIEYQVKDVLTPLFKIIG
   MV (AB012948)   (51) GLLAIAGIRLHRAAIYTAEIHKSLSTNLDVTNSIEHQVKDVLTPLFKIIG
   MV (AF266288)   (51) GLLAIAGIRLHRAAIYTAEIHKSLSTNLDVTNSIEHQVKDVLTPLFKIIG
   MV (AF266286)   (51) GLLAIAGIRLHRAAIYTAEIHKSLSTNLDVTNSIEHQVKDVLTPLFKIIG
   MV (AF266289)   (51) GLLAIAGIRLHRAAIYTAEIHKSLSTNLDVTNSIEHQVKDVLTPLFKIIG
  CDV (AF164967)   (51) ALLAITGVRFHQVSTSNMEFSRLLKEDMEKSEAVHHQVIDVLTPLFKIIG
  CDV (AY466011)   (51) ALLSITGIRFHKVSTSNMEFSRLLKEDMEKSEAVHHQVIDVLTPLFKIIG
  CDV (AF378705)   (51) ALLAITGVRFHQVSTSNMEFSRLLKEDMEKSEAVHHQVIDVLTPLFKIIG
  CDV (AF305419)   (51) ALLAITGVRFHQVSTSNMEFSRLLKEDMEKSEAVHHQVIDVLTPLFKIIG

                       101                                                150
 PPRV (AJ512718)  (101) DEVGIRIPQKFSDLVKFISDKIKFLNPDREYDFRDLRWCMNPPERVKINF
PPRV (NC_006383)  (101) DEVGIRIPQKFSDLVKFISDKIKFLNPDREYDFRDLRWCMNPPERVKINF
   PPRV (X74443)  (101) DEVGIRIPQKFSDLVKFISDKIKFLNPDREYDFRDLRWCMNPPERVKINF
    RPV (X98291)  (101) DEVGLRTPQRFTDLTKFISDKIKFLNPDKEYDFRDINWCINPPERIKIDY
    RPV (Z30697)  (101) DEVGLRTPQRFTDLTKFISDKIKFLNPDKEYDFRDINWCINPPERIKIDY
   MV (AB012948)  (101) DEVGLRTPQRFTDLVKFISDKIKFLNPDREYDFRDLTWCINPPERIKLDY
   MV (AF266288)  (101) DEVGLRTPQRFTDLVKFISDKIKFLNPDREYDFRDLTWCINPPERIKLDY
   MV (AF266286)  (101) DEVGLRTPQRFTDLVKFISDKIKFLNPDREYDFRDLTWCINPPERIKLDY
   MV (AF266289)  (101) DEVGLRTPQRFTDLVKFISDKIKFLNPDREYDFRDLTWCINPPERIKLDY
  CDV (AF164967)  (101) DEIGLRLPQKLNEIKQFILQKTNFFNPNREFDFRDLHWCINPPSKIKVNF
  CDV (AY466011)  (101) DEIGLRLPQKLNEIKQFILQKTNFFNPNREFDFRDLHWCINPPSKVKVNF
  CDV (AF378705)  (101) DEIGLRLPQKLNEIKQFILQKTNFFNPNREFDFRDLHWCINPPSKVKVNF
  CDV (AF305419)  (101) DEIGLRLPQKLNEIKQFILQKTNFFNPNREFDFRDLHWCINPPSTVKVNF

                       151                                                200
 PPRV (AJ512718)  (151) DQFCEYKAAVKSIEHIFESPLNKSKKLQSLTLGPGTGCQGRTVTRAHFSE
PPRV (NC_006383)  (151) DQFCEYKAAVKSIEHIFESPLNKSKKLQSLTLGPGTGCLGRTVTRAHFSE
   PPRV (X74443)  (151) DQFCEYKAAVKSVEHIFESSLNRSERLRLLTLGPGTGCLGRTVTRAQFSE
    RPV (X98291)  (151) DQYCAHTAAEDLITMLVNSSLTGTTVLRTSLVNLGRNCTGPTTTKGQFSN
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   MV (AF266288)  (151) DQYCADVAAEELMNALVNSTLLETRTTNQFLAVSKGNCSGPTTIRGQFSN
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   MV (AF266289)  (151) DQYCADVAAEELMNALVNSTLLETRTTNQFLAVSKGNCSGPTTIRGQFSN
  CDV (AF164967)  (151) TNYCDTIGIRKSIASAANPILLSALSGGRGDIFPPYRCSGATTSVGRVFP
  CDV (AY466011)  (151) TNYCDTIGIRKSIASAANPILLSALSGSRSDIFPPYRCSGATTSVGKVFP
  CDV (AF378705)  (151) TNYCESIGIRKAIASAANPILLSALSGGRSDIFPPHRCSGATTSVGKVFP
  CDV (AF305419)  (151) TNYCESIGIRKAIASAANPILLSALSGGRGDIFPPHRCSGATTSVGKVFP
```

```
                          201                                                250
 PPRV (AJ512718)  (201) LTLTLMDLDLEMKHNVSSVFTVVEEGLFGRTYTVWRSDARDPSTDPGIGH
PPRV (NC_006383)  (201) LTLTLMDLDLEMKHNVSSVFTVVEEGLFGRTYTVWRSDARDPSTDLGIGH
   PPRV (X74443)  (201) LTLTLMDLDLEIKHNVSSVFTVVEEGLFGRTYTVWRSDTGKPSTSPGIGH
    RPV (X98291)  (201) ISLTLSGIYSGRGYNISSMITITGKGMYGSTYLVGKYNQRARRPSIVWQQ
    RPV (Z30697)  (201) ISLTLSGIYSGRGYNISSMITITGKGMYGSTYLVGKYNQRARRPSKVWHQ
    MV (AB012948)  (201) MSLSLLDLYLSRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKGSELSQLS
    MV (AF266288)  (201) MSLSLLDLYLSRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQLS
    MV (AF266286)  (201) MSLSLLDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQLS
    MV (AF266289)  (201) MSLSLLDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQLS
   CDV (AF164967)  (201) LSVSLSMSLISRTSEIINMLTAISDGVYGKTYLLVPDYIEGGFDT----Q
   CDV (AY466011)  (201) LSVSLSMSLISRTSVIINMLTAISDGVYGKTYLLVPDDIEREFDT----Q
   CDV (AF378705)  (201) LSVSLSMSLISRTSEIINMLTAISDGVYGKTYLLVPDDIEREFDT----Q
   CDV (AF305419)  (201) LSVSLSMSLISRTSEVINMLTAISDGVYGKTYLLVPDDIEREFDT----R

                          251                                                300
 PPRV (AJ512718)  (251) FLRVFEIGLVRDLGLGPPVFHMTNYLTVNMSDDYRRCLLAVGELKLTALC
PPRV (NC_006383)  (251) FLRVFEIGLVRDLGLGPPVFHMTNYLTVNMSDDYRRCLLAVGELKLTALC
   PPRV (X74443)  (251) FLRVFEIGLVRDLELGAPIFHMTNYLTVNMSDDYRSCLLAVGELKLTALC
    RPV (X98291)  (251) DYRVFEVGIIRELGVGTPVFHMTNYLELPRQPELETCMLALGESKLAALC
    RPV (Z30697)  (251) DYRVFEVGIIRELGVGTPGFHMTNYLELPRQPELETCMLALGESKLAALC
    MV (AB012948)  (251) MHRVFEVGVIRNPGLGAPVFHMTNYFEQSVSNDFSNCMVALGELKFAALC
    MV (AF266288)  (251) MYRVFEVGVIRNPGLGAPVFHMTNYLEQPVSNDLSNCMVALGELKLAALC
    MV (AF266286)  (251) MYRVFEVGVIRNPGLGAPVFHMTNYLEQPVSNDLSNCMVALGELKLAALC
    MV (AF266289)  (251) MYRVFEVGVIRNPGLGAPVFHMTNYLEQPVSNDLSNCMVALGELKLAALC
   CDV (AF164967)  (247) KIRVFEIGFIKRWLNDMPLLQTTNYMVLPENSKAKVCTIAVGELTLASLC
   CDV (AY466011)  (247) EIRVFEIGFIKRWLNDMPLLQTTNYMVLPENSKAKVCTIAVGELTLASLC
   CDV (AF378705)  (247) EIRVFEIGFIKRWLNDMPLLQTTNYMVLPENSKAKVCTIAVGELTLASLC
   CDV (AF305419)  (247) EIRVFEIGFIKRWLNDMPLLQTTNYMVLPKNSKAKVCTIAVGELTLASLC

                          301                                                350
 PPRV (AJ512718)  (301) TSSETVTLSERGVPKRKPLVVVILNLAGPTLGGELYSVLPTSDLMVEKLY
PPRV (NC_006383)  (301) SSSETVTLGERGVPKREPLVVVILNLAGPTLGGELYSVLPTSDLMVEKLY
   PPRV (X74443)  (301) TPSETVTLSESGVPKREPLVVVILNLAGPTLGGELYSVLPTTDPTVEKLY
    RPV (X98291)  (301) LADSPVALHYGRVGDDNKIRFVKLGVWASPADRDTLATLSAIDPTLDGLY
    RPV (Z30697)  (301) LADSPVALHYGRVGDDNKIRFVKLGVWASPADRDTLATLSAIDPTLDGLY
    MV (AB012948)  (301) HREDSITIPYQGSGKGVSFQLVKLGVWKSPTDMQSWVPLSTDDPVIDRLY
    MV (AF266288)  (301) HGEDSITIPYQGSGKGVSFQLVKLGVWKSPTDMQSWVPLSTDDPVIDRLY
    MV (AF266286)  (301) HREDSITIPYQGSGKGVSFQLVKLGVWKSPTDMQSWVTLSTDDPVIDRLY
    MV (AF266289)  (301) HGEDSITIPYQGSGKGVSFQLVKLGVWKSPTDMQSWVPLSTDDPVIDRLY
   CDV (AF164967)  (297) VDESTVLLYHDSDGSQDGILVVTLGIFGATPMDQVEEVIPVAHPSVEKIH
   CDV (AY466011)  (297) VDESTVLLYHDSRGSQDGILVVTLGIFGATPMDHIEEVIPVAHPSMEKIH
   CDV (AF378705)  (297) VEESTVLLYHDSSGSQDGILVVTLGIFWATPMDHIEEVIPVAHPSMEKIH
   CDV (AF305419)  (297) VEESTVLLYHDSSGSQDGILVVTLGIFWATPMDHIEEVIPVAHPSMKKIH

                          351                                                400
 PPRV (AJ512718)  (351) LSSHRGIIKDNEANWVVPSTDVRDLQNKGECLVEACKTRPPSFCNGTGSG
PPRV (NC_006383)  (351) LSSHRGIIKDDEANWVVPSTDVRDLQNKGECLVEACKTRPPSFCNGTGSG
   PPRV (X74443)  (351) LSSHRGIIKDNEANWVVPSTDVRDLQNKGECLVEACKTRPPSFCNGTGIG
    RPV (X98291)  (351) ITTHRGIIAAGTAIWAVPVTRTDDQVKMGKCRLEACRDRPPPFCNSTDWE
    RPV (Z30697)  (351) ITTHRGIIAAGTAIWAVPVTRTDDQVKMGKCRLEACRDRPPPFCNSTDWE
    MV (AB012948)  (351) LSSHRGVIADNQAKWAVPTTRTDDKLRMETCFQQACKGKIQALCENPEWA
    MV (AF266288)  (351) LSSHRGVIADNQAKWAVPTTRTDDKLRMETCFQQACKGKIQALCENPEWA
    MV (AF266286)  (351) LSSHRGVIADNQAKWAVPTTRTDDKLRMETCFQQACKGKIQALCENPEWA
    MV (AF266289)  (351) LSSHRGVIADNQAKWAVPTTRTDDKLRMETCFQQACKGKIQALCENPEWA
   CDV (AF164967)  (347) ITNHRGFIKDSIATWMVPALVSEKQEEQKNCLESACQRKSYPMCNQTSWE
   CDV (AY466011)  (347) ITNHRGFIKDSIATWMVPALASEKQEEQKGCLESACQRKTYPMCNQTSWE
   CDV (AF378705)  (347) ITNHRGFIKDSIATWMVPALASEKQEEQKGCLESACQRKTYPMCNQTSWE
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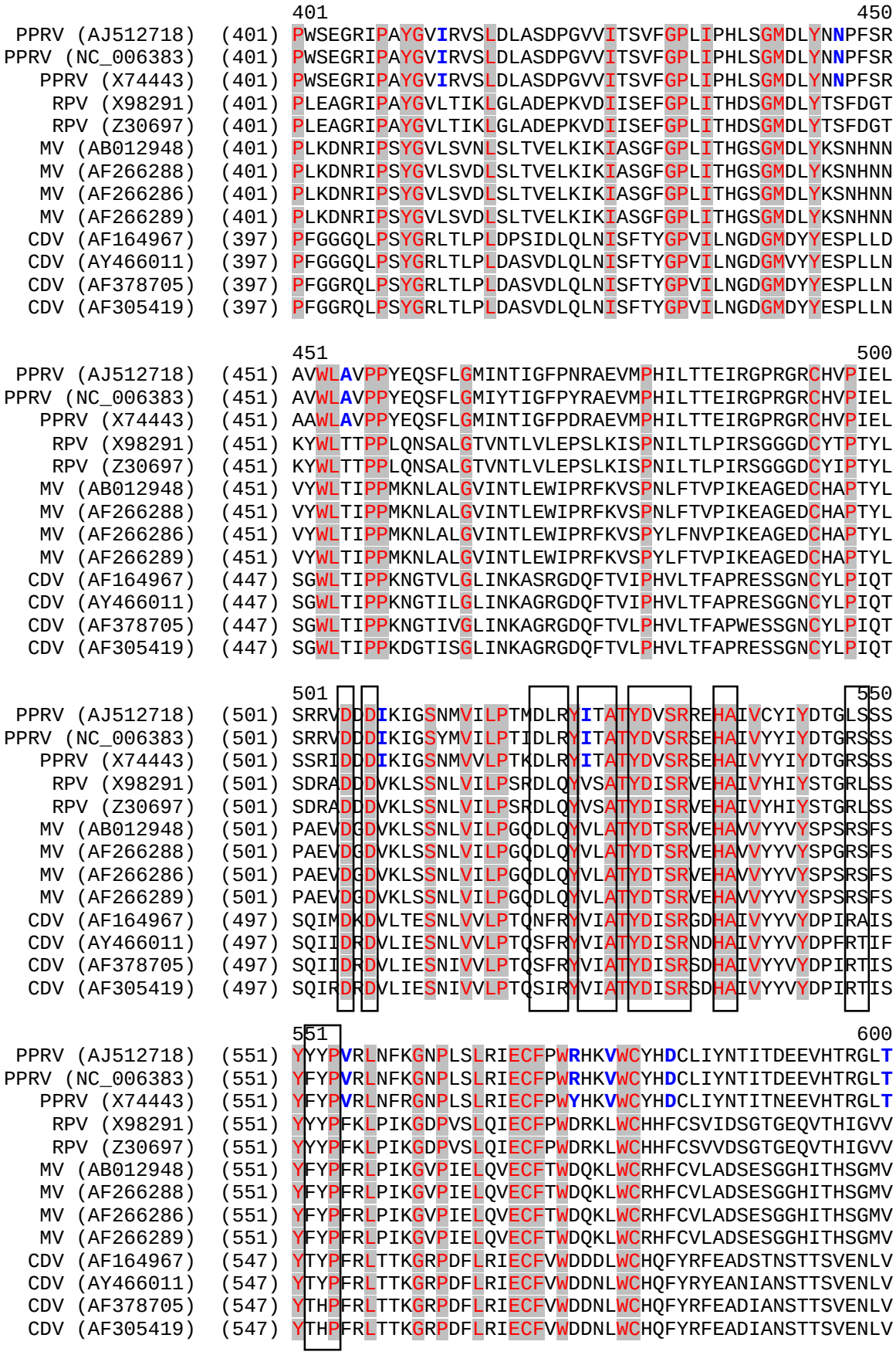
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PPRV (X74443) (401) PWSEGRIPAYGVIRVSLDLASDPGVVITSVFGPLIPHLSGMDLYNNPFSR
RPV (X98291) (401) PLEAGRIPAYGVLTIKLGLADEPKVDIISEFGPLITHDSGMDLYTSFDGT
RPV (Z30697) (401) PLEAGRIPAYGVLTIKLGLADEPKVDIISEFGPLITHDSGMDLYTSFDGT
MV (AB012948) (401) PLKDNRIPSYGVLSVNLSLTVELKIKIASGFGPLITHGSGMDLYKSNHNN
MV (AF266288) (401) PLKDNRIPSYGVLSVDLSLTVELKIKIASGFGPLITHGSGMDLYKSNHNN
MV (AF266286) (401) PLKDNRIPSYGVLSVDLSLTVELKIKIASGFGPLITHGSGMDLYKSNHNN
MV (AF266289) (401) PLKDNRIPSYGVLSVDLSLTVELKIKIASGFGPLITHGSGMDLYKSNHNN
CDV (AF164967) (397) PFGGGQLPSYGRLTLPLDPSIDLQLNISFTYGPVILNGDGMDYYESPLLD
CDV (AY466011) (397) PFGGGQLPSYGRLTLPLDASVDLQLNISFTYGPVILNGDGMVYYESPLLN
CDV (AF378705) (397) PFGGRQLPSYGRLTLPLDASVDLQLNISFTYGPVILNGDGMDYYESPLLN
CDV (AF305419) (397) PFGGRQLPSYGRLTLPLDASVDLQLNISFTYGPVILNGDGMDYYESPLLN
451 500
PPRV (AJ512718) (451) AVWLAVPPYEQSFLGMINTIGFPNRAEVMPHILTTEIRGPRGRCHVPIEL
PPRV (NC_006383) (451) AVWLAVPPYEQSFLGMIYTIGFPYRAEVMPHILTTEIRGPRGRCHVPIEL
PPRV (X74443) (451) AAWLAVPPYEQSFLGMINTIGFPDRAEVMPHILTTEIRGPRGRCHVPIEL
RPV (X98291) (451) KYWLTTPPLQNSALGTVNTLVLEPSLKISPNILTLPIRSGGGDCYTPTYL
RPV (Z30697) (451) KYWLTTPPLQNSALGTVNTLVLEPSLKISPNILTLPIRSGGGDCYIPTYL
MV (AB012948) (451) VYWLTIPPMKNLALGVINTLEWIPRFKVSPNLFTVPIKEAGEDCHAPTYL
MV (AF266288) (451) VYWLTIPPMKNLALGVINTLEWIPRFKVSPNLFTVPIKEAGEDCHAPTYL
MV (AF266286) (451) VYWLTIPPMKNLALGVINTLEWIPRFKVSPYLFNVPIKEAGEDCHAPTYL
MV (AF266289) (451) VYWLTIPPMKNLALGVINTLEWIPRFKVSPYLFTVPIKEAGEDCHAPTYL
CDV (AF164967) (447) SGWLTIPPKNGTVLGLINKASRGDQFTVIPHVLTFAPRESSGNCYLPIQT
CDV (AY466011) (447) SGWLTIPPKNGTILGLINKAGRGDQFTVIPHVLTFAPRESGGNCYLPIQT
CDV (AF378705) (447) SGWLTIPPKNGTIVGLINKAGRGDQFTVLPHVLTFAPWESSGNCYLPIQT
CDV (AF305419) (447) SGWLTIPPKDGTISGLINKAGRGDQFTVLPHVLTFAPRESSGNCYLPIQT
501 550
PPRV (AJ512718) (501) SRRVDDDIKIGSNMVILPTMDLRYITATYDVSRREHAIVCYIYDTGLSSS
PPRV (NC_006383) (501) SRRVDDDIKIGSYMVILPTIDLRYITATYDVSRSEHAIVYYIYDTGRSSS
PPRV (X74443) (501) SSRIDDDIKIGSNMVVLPTKDLRYITATYDVSRSEHAIVYYIYDTGRSSS
RPV (X98291) (501) SDRADDDVKLSSNLVILPSRDLQYVSATYDISRVEHAIVYHIYSTGRLSS
RPV (Z30697) (501) SDRADDDVKLSSNLVILPSRDLQYVSATYDISRVEHAIVYHIYSTGRLSS
MV (AB012948) (501) PAEVDGDVKLSSNLVILPGQDLQYVLATYDTSRVEHAVVYYVYSPSRSFS
MV (AF266288) (501) PAEVDGDVKLSSNLVILPGQDLQYVLATYDTSRVEHAVVYYVYSPGRSFS
MV (AF266286) (501) PAEVDGDVKLSSNLVILPGQDLQYVLATYDTSRVEHAVVYYVYSPSRSFS
MV (AF266289) (501) PAEVDGDVKLSSNLVILPGQDLQYVLATYDTSRVEHAVVYYVYSPSRSFS
CDV (AF164967) (497) SQIMDKDVLTESNLVVLPTQNFRYVIATYDISRGDHAIVYYVYDPIRAIS
CDV (AY466011) (497) SQIIDRDVLIESNLVVLPTQSFRYVIATYDISRNDHAIVYYVYDPFRTIF
CDV (AF378705) (497) SQIIDRDVLIESNIVVLPTQSFRYVIATYDISRSDHAIVYYVYDPIRTIS
CDV (AF305419) (497) SQIRDRDVLIESNIVVLPTQSIRYVIATYDISRSDHAIVYYVYDPIRTIS
551 600
PPRV (AJ512718) (551) YYYPVRLNFKGNPLSLRIECFPWRHKVWCYHDCLIYNTITDEEVHTRGLT
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PPRV (X74443) (551) YFYPVRLNFRGNPLSLRIECFPWYHKVWCYHDCLIYNTITNEEVHTRGLT
RPV (X98291) (551) YYYPFKLPIKGDPVSLQIECFPWDRKLWCHHFCSVIDSGTGEQVTHIGVV
RPV (Z30697) (551) YYYPFKLPIKGDPVSLQIECFPWDRKLWCHHFCSVVDSGTGEQVTHIGVV
MV (AB012948) (551) YFYPFRLPIKGVPIELQVECFTWDQKLWCRHFCVLADSESGGHITHSGMV
MV (AF266288) (551) YFYPFRLPIKGVPIELQVECFTWDQKLWCRHFCVLADSESGGHITHSGMV
MV (AF266286) (551) YFYPFRLPIKGVPIELQVECFTWDQKLWCRHFCVLADSESGGHITHSGMV
MV (AF266289) (551) YFYPFRLPIKGVPIELQVECFTWDQKLWCRHFCVLADSESGGHITHSGMV
CDV (AF164967) (547) YTYPFRLTTKGRPDFLRIECFVWDDDLWCHQFYRFEADSTNSTTSVENLV
CDV (AY466011) (547) YTYPFRLTTKGRPDFLRIECFVWDDNLWCHQFYRYEANIANSTTSVENLV
CDV (AF378705) (547) YTHPFRLTTKGRPDFLRIECFVWDDNLWCHQFYRFEADIANSTTSVENLV
CDV (AF305419) (547) YTHPFRLTTKGRPDFLRIECFVWDDNLWCHQFYRFEADIANSTTSVENLV

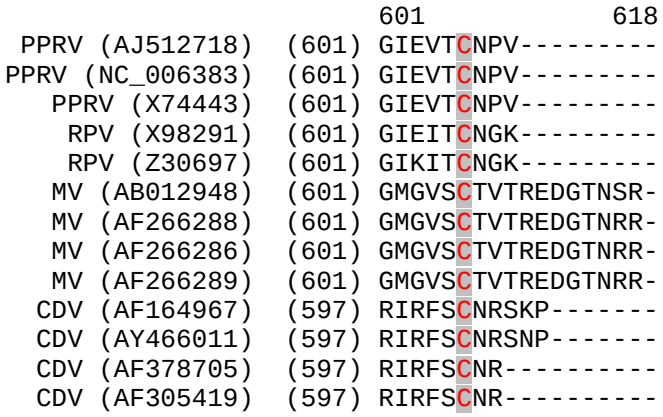
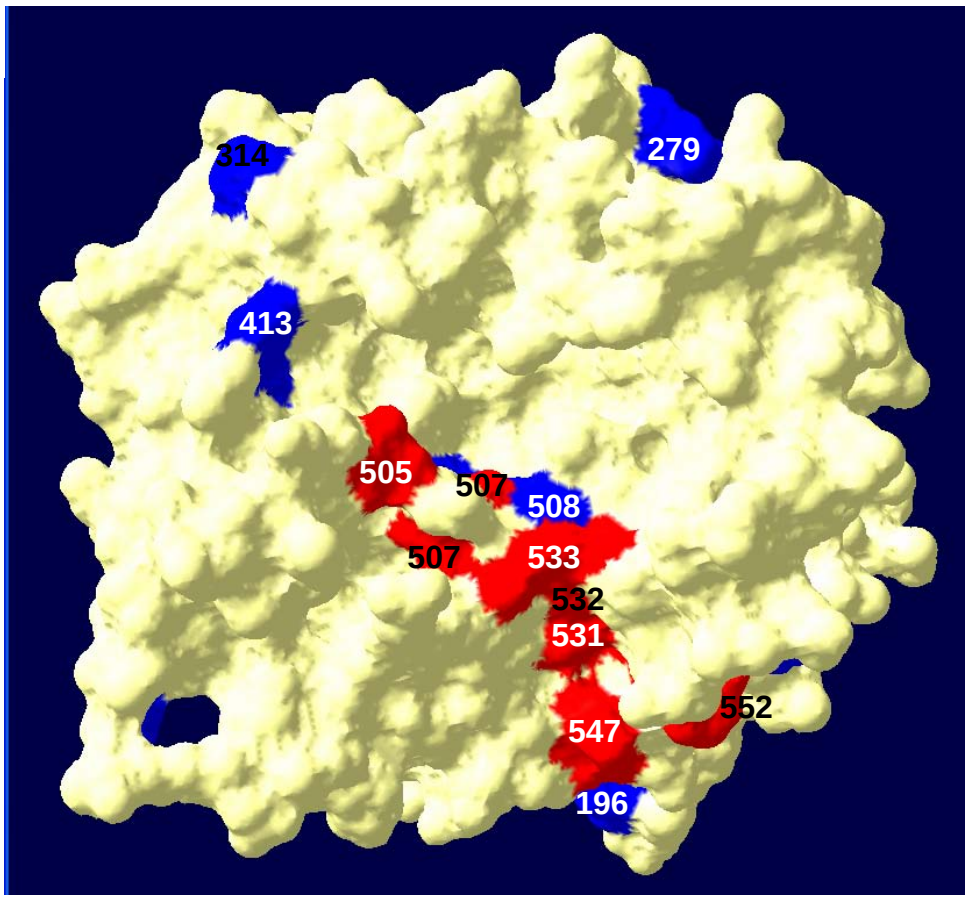


Fig. 5 Analysis of H amino-acid sequence of PPRV strains and comparison with other vaccine (vac) and wild type (wt) strains of *Morbillivirus*. Red letters in grey boxes display the identical amino-acid residues. Black boxes identify critical residues for H-MV/SLAM interaction (positions 505, 507, 521-523, 525-527, 529-533, 536-537, 547-548, 552-554). Blue letters indicate PPRV specific mutations compared to other morbilliviruses (27 positions). Accession numbers used in this figure are PPRV (vac: X74443, wt: AJ512718 and NC_006383), RPV (vac: Z30697, wt: X98291), MV (vac: AF266286 and AF266289, wt: AF266288 and AB012948), CDV (vac: AF305419 and AF378705, wt: AF164967 and AY466011).

(a)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413
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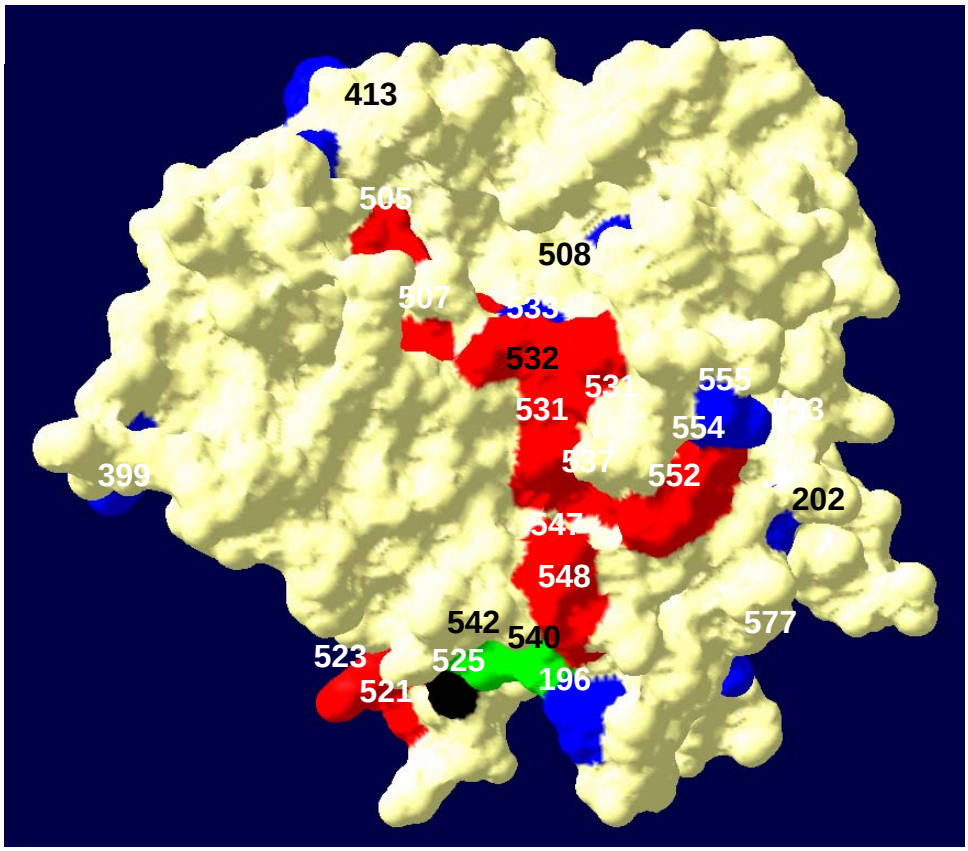
196

(b)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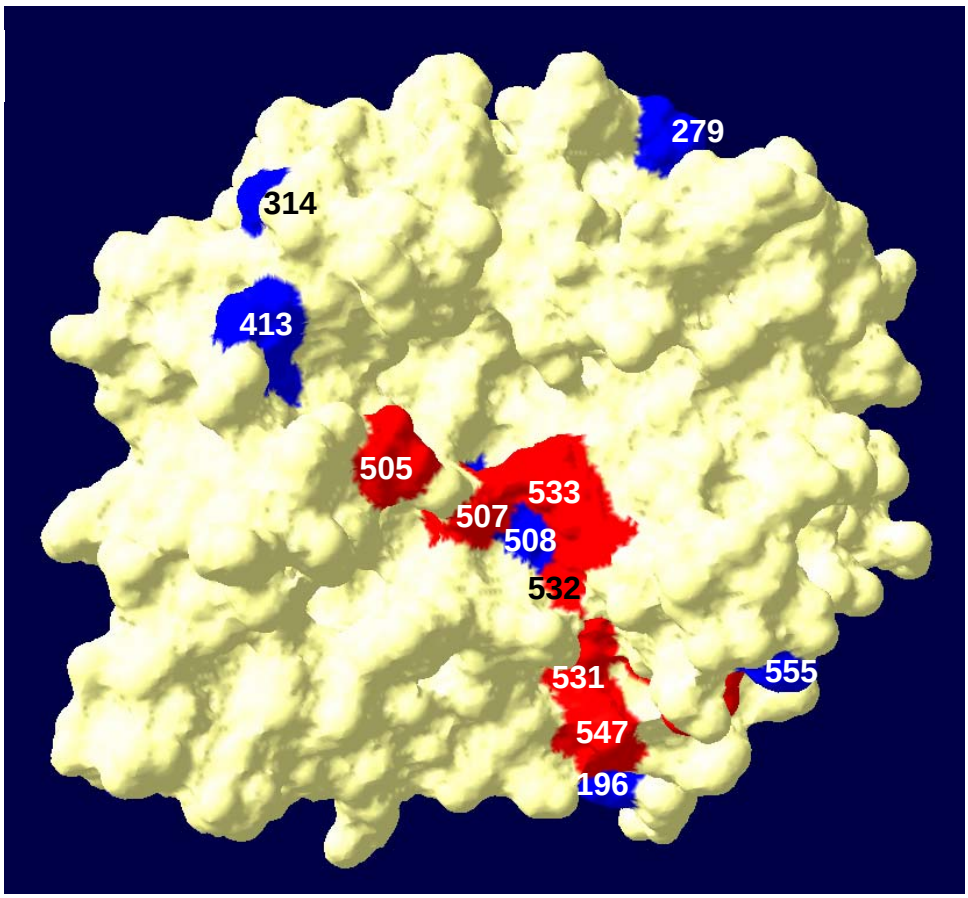
(c)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(d)

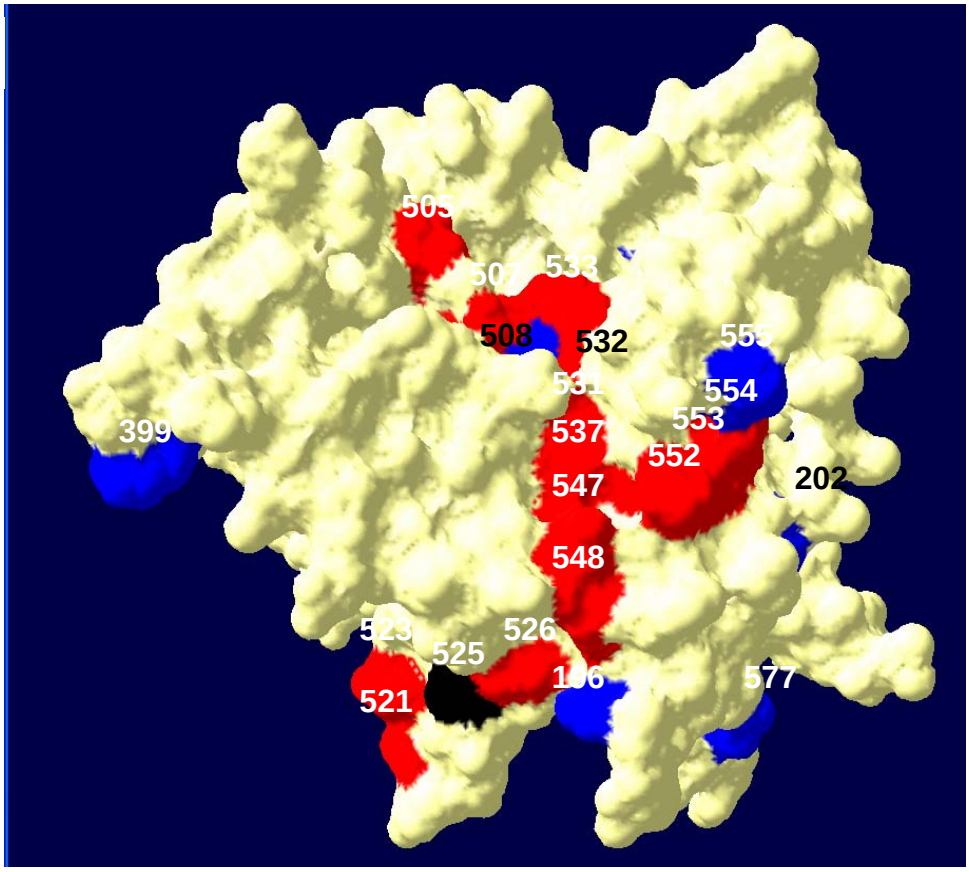
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Fig. 6 3D surface models of the globular head of the H protein of PPRV (a and b) in comparison with MV (c and d). Top views are shown on (a) and (c), while (b) and (d) are side views rotated by about 315° around the x axis. Red surfaces display the critical residues identified on the H-MV protein for its interaction with the SLAM (CD150) receptor of B95a (Masse *et al*, 2004). Blue surfaces show the amino-acids specifically mutated on the H-PPRV compared to the other morbillivirus. The black surface is a critical residue for SLAM receptor interaction that was found only mutated on the H-PPRV. Green surfaces represent two amino-acids in the H-PPRV that land in place of the amino-acid 225 in the H-MV.

Difference in virulence may not result exclusively on the host or cell susceptibility. Once entered in the cells, the virus may not necessarily find the necessary environment for an efficient replication. This could explain a diffence in the clinical outcome. Inside the cells, the major biological processes on which relies the virus replication, involve three proteins tighly associated with the nucleic acid to form the ribonucleic complex. The function of this complex is dependent on the presence of particular inverted terminal sequences at the genome extremities. These sequences are named genome promoter (3' – 5', genome sens) and antigenome promoter (5' – 3' genome antisens). The first one is responsible for the mRNA transcription and the production of the positive full genome RNA that serves as a template for genome replication. The antigenome promoter is responsible for the production of new genome molecule. Promoter sequences are conserved regions of the gene because of the essential role they play in transcription and replication. Nucleotide mutations in these regions may result in a loss of virulence, at least between wild type virus and cell culture adapted vaccine strain. We were also interested to see if differences in these sequences could be seen between strains producing disease in goats and strains producing disease in sheep. In this study, base changes that may contribute to viral attenuation were investigated by comparative analysis of the GP and AGP from field strains affecting sheep or goats and a vaccinal. The promoter sequences were also compared with those from other members of the *Morbillivirus* genus and the family *Paramyxoviridae* to assess possible shared critical residues for virulence and the phylogenetic relationships between all these viruses.

Chapter 5

Anticle 3* : Submitted to *Journal of General Virology

Sequence analysis of the genome and anti-genome promoters of peste des petits ruminants virus (PPRV), comparison with other *Morbillivirus*

G. Abraham[1], G. Libeau[1], A. Diallo[2], S. Thomassin[1],C. Minet[1], M Yami[1], O. Kwiatek[1], O. Friedgut[3], E. Albina[1],*

1 : CIRAD- EMVT, TA30/G, Campus International de Baillarguet, 34398 Montpellier, Cedex, 5, FRANCE.
2 : FAO/IAEA Agriculture and Biotechnology Laboratory, IAEA Laboratories, Animal Production Unit, A-2444, Seibersdorf, Austria
3 : Kimron Veterinary Institute, Beit Dagan 50250, Israel.

Abstract

Genome and antigenome promoters (GP and AGP) of the peste des petits ruminants (PPR) virus, amplified from different pathological samples of sheep and goat origins, were sequenced and compared with corresponding sequences of PPR vaccine strain and other morbilliviruses. Alignment of GP sequences revealed six nucleotide changes at positions 5, 12, 26, 36, 42 and 81, and one nucleotide mutation in AGP at position 15842 between PPR vaccine and field strains. Mutation 26 was clearly linked with the attenuated phenotype of the vaccine strains of PPRV, rinderpest virus (RPV), measles virus (MV) and canine distemper virus (CDV) as well. Mutations 5 and 12 were only seen on the PPRV and RPV vaccine strains and 4 other mutations, 36, 42, 81 and 15842 were seen only on the PPRV vaccine strain sequence. Interestingly, the field strains of PPRV were the only ones showing a residue U at position 36 instead of C for other morbilliviruses and the PPR vaccine strain. These nucleotide mutations, or some of them, may influence gene expression by changing the interaction of the leader with the viral polymerase or with a cellular protein involved in the modulation of the gene replication/transcription. As such they may be some of the candidates involved in the attenuation of PPRV. In spite of differences in virulence between goats and sheep reported in the field, there had been 100% homology of sequences in clinical samples of nasal swabs, lung and lymph nodes from both species. However, a higher heterogeneity within strains isolated from different areas was evidenced and accounted for a relevant phylogenetic clustering according to the geographic origin. It was also shown that the promoter sequences, although very short compared to the full genome, could make relevant phylogenetic tree of *Paramyxoviridae*.

Key words: leader, morbillivirus, peste des petits ruminants (PPR), promoter, rinderpest (RP), trailer

*Corresponding author: Dr Genevieve Libeau; Genevieve.libeau@cirad.fr

Introduction

Peste des petits ruminants (PPR) is an acute and highly contagious viral disease of domestic and wild small ruminants, which is characterised by high fever, ocular and nasal discharge, pneumonia, necrosis and ulceration of the mucous membrane and inflammation of the gastro-intestinal tract leading to severe diarrhoea and death (Gibbs *et al.*, 1979). PPR is caused by a single strand non-segmented negative RNA virus which belongs to the family *Paramyxoviridae*, genus *Morbillivirus* which also includes measles virus, rinderpest virus (RPV), canine-distemper virus, phocine-distemper virus, and dolphin and porpoise morbilliviruses (Barrett *et al.*, 1993a). All morbilliviruses are related serologically, and sequence data shows that there is a high degree of homology at the sequence level. The genome contains six tandemly arranged transcription units encoding six structural proteins, the surface glycoproteins F and H, the nucleocapsid (N), the matrix (M), the polymerase or large (L) and the polymerase-associated (P) proteins. The cistron directing the synthesis of this later protein is encoding the virus non-structural proteins C and V by the use of two other open reading frames (ORF) of the messengers. The gene order is 3'N-P-M-F-H-L5', as determined by transcriptional mapping (Dowling *et al.*, 1986). The genome is flanked by extragenic sequences at the 3' and 5' ends, referred to as the leader (52 nucleotides) and trailer (37 nucleotides), respectively. For viruses of the family *Paramyxoviridae*, the genome promoter (GP) includes 107 nucleotides comprising the leader sequence and the adjacent non-coding region of the N gene at the 3' end of the negative-strand. The antigenome promoter (AGP) includes 109 nucleotides that encompass the trailer sequence and the proximal untranslated region of the L gene. Both the GP and the AGP contains the polymerase binding sites and the RNA encapsidation signals for the replication of the full genome while the production of messengers RNA is a function of the GP (Murphy *et al.*, 1998; Murphy and Parks 1999; Tapparel *et al.*, 1998; Walpita, 2004). Therefore nucleotide changes in the GP and AGP of the genome which affect the replication/transcription of the RNA may have an impact on the virulence of the virus. Comparison of sequence data from

vaccinal and virulent strains of rinderpest and measles viruses identified nucleotide changes throughout the genome. Those located in the GP and AGP are among the potential attenuation of the virus (Parks *et al*., 2001; Baron *et al*., 1996; Banyard *et al*, 2005). In this paper, evidence of base changes between an attenuated vaccine strain and virulent PPRV strains from the field was obtained by the comparative analysis of the GP and AGP sequences. We used also those sequences to identify the relationship between the different samples we analysed and compared these data with those previously reported for the F protein gene sequence (Shaila *et al*, 1996; Dhar *et al*., 2002). The promoter sequences were also compared with those from other members of the *Morbillivirus* genus for the identification of shared attenuation genetic markers, and of the *Paramyxoviridae* family for their phylogenetic relationships.

Materials and Methods

The samples

The samples were obtained either as nasal swabs or as post-mortem tissues submitted for confirmatory diagnosis from clinical diseases in goats and sheep (Table 1). A set of samples from goats and sheep collected in Israel and covering several years of collection was included in order to assess the molecular variation of the promoters within a given region. All samples were processed using standard methods (Couacy-Hymann *et al*., 2002) and one hundred microlitres of the sample solution were used for the RNA extraction. Vaccine strain of PPR virus (Diallo *et al*,. 1989) was grown in Vero cells. Cell growth medium was Eagles MEM supplemented with 10% fœtal bovine serum (FBS) and 1% of a mixed antibiotic solution (Gibco, Life Technologies, UK). Infected cells and the supernatant were harvested at 75% of the cytopathic effect (CPE), and frozen at -70°C until RNA extraction.

RNA extraction

RNA was extracted as described by Couacy-Hymann and others (2002). Briefly, nine hundred µl lysis solution (containing guanidine isothiocyanate, β-mercaptoethanol, sarcosyl and sodium citrate)

and 100 µl sample suspension were mixed. The tube was then centrifuged for 3 minutes at 10,000 rpm. The supernatant was used in subsequent steps. One volume of 70% ethanol was mixed by pipetting and the mixed solution was extracted by rapid spinning at 10,000 rpm through RNeasy mini spin column (Qiagen, USA). The supernatant was discarded and the pellet was washed three times with washing solution (Ethanol 50%, Tris-Hcl 10mM pH7.4-7.6, EDTA 1mM, NaCl 50mM). The pellet was eluted in 50 µl of DEPC-treated water. One µl of RNase inhibitor (10U/µl) (Amersham,UK) was added to the RNA solution and frozen at –70°C until used.

Single stranded cDNA synthesis and PCR for virus detection and sequencing

For the detection of PPRV in pathological samples, the RNA was denatured at 65°C for ten minutes. Reverse transcription (RT) reaction was carried out in a 250 µl PCR tube as follows : 8 µl of the extracted RNA was mixed with 1µl of DTT, 1µl of random primer pd(N)6 and 5 µl of First strand cDNA bulk (Amersham, UK). The mixture was incubated at 37°C for 1h. PCR was conducted using primers designed by Couacy-Hymann *et al.* (2002) to amplify fragments of the PPRV N gene. The forward (5'-CAAGCCAAGGATTGCAGAAATGA-3') and reverse (5'-AATTGAGTTCTCTAGAATCACCAT-3') primers were universal *Morbillivirus*, while the forward (5'-GTCTCGGAAATCGCCTCACAG-3') and reverse (5'-CCTCCTCCTG GTCCTCCAGAA-3') primers were PPR specific. In a 250µl PCR microtube, cDNA was mixed with the following reagents: 5 µl of dNTP mixture (200mM), 5µl of 10X Taq buffer, 5 µl of primers (5 pmol/µl), 36 µl of water and 1 µl of Taq polymerase (1.25U/µl) (Qiagen, USA). Once all the reagents were mixed, the tube was placed into the Gene Amp PCR system 2400 (Applied Biosystem, USA). The amplification was carried out according to the following programme: an initial heating step 95°C for 5 min followed by denaturation at 94°C for 2 min and 35 cycles of 94°C for 30s, annealing at 55°C for 30 s, extension at 72 °C for 2 minutes.

The full genome sequence of PPRV vaccine strain Nigeria 75/1 was determined in our laboratory (accession numbers: X74443, Z37017, Z47977, Z81358).The 3' and 5' ends of the PPRV vaccine

strain was amplified from the virus infected cell total RNA using the 5´ RACE System for Rapid Amplification of cDNA Ends kit following instructions of the supplier (Invitrogen, USA). Primers used in those reactions were designed based on the N gene (5'-ATCATCTGTGATCCGCTGTATCAAT-3') for the genome 3' end cloning and the L gene (5'-TCCACCATGATGTTGCCTCAGG-3') for the genome 5'. For the amplification of the genome and antigenome promoters of other PPRV strains, the same primers were used in conjunction with new primers which were designed at the genome extremities based on the vaccine strain leader and trailer sequences previously determined, 5'-ACCAGACAAAGCTGGGTAAGGATA-3' and 5'-ACCAGACAAAGCTGGGTAAGGATA-3', respectively. Two hundred pmol of each primer were used in a 50 µl PCR reaction mixture. The amplification conditions were the same as indicated previously with exception of changes in annealing temperature of 52°C for 30 seconds in the trailer PCR cycle.

Analysis of PCR- amplified products

Ten microlitres of the amplicon were analysed by electrophoresis on a gel which was made of 1.5% agarose in Tris-borate-EDTA (TBE) buffer (0.089M Tris base, 0.089M boric acid and 0.002M EDTA, pH 8.3). The gel was stained with ethidium bromide and the DNA was visualised by UV fluorescence and photographed.

Determination of GP and AGP sequences

PCR products were purified on columns (Qiaquick PCR Purification kit, Qiagen, USA) and directly sequenced. Cycle sequencing was performed using dye-labeled terminators and Taq DNA polymerase (Applied Biosystems, USA), followed by analysis on an ABI Prism 377 automatic sequencer (Applied Biosystems, USA). Analysis of sequence results was performed using Vector NTI-9 package (Informax Inc., USA). Sequence alignments were done using CLUSTAL W included in this package. For the analysis of nucleotide changes, 6 PPRV sequences from Genbank (Table 2), including one vaccine strain sequence, were first considered because the 5'end of their

GP and the 3'end of their AGP have been completed. Subsequently, interesting positions identified previously were confirmed on partial promoter sequences (primers excluded) of our 12 additional virulent strains detected in field samples (coded strains in Table 1). All Israeli strains were sequenced but only one from each collection year and the divergent ones within a given year were used. Phylogenetic analysis was then carried out using a criterion of neighbourhood based on the principle of parsimony (Saitou and Nei, 1987). Dissimilarities and distances between the sequences were first determined using the Darwin 5 software (Perrier *et al.,* 2003). Trees were then generated with the TreeCon MATRIXW program (Van de peer and De Watchter, 1993) included in Darwin5. Tree construction was based on the unweighted Neighbor-Joining method proposed by Gascuel (1997). Bootstraps were determined on 1000 replicates.

Results and Discussion

Base substitutions on the GP and AGP between the virulent strains of PPRV and the vaccine strain

In the cycle of infection, the RNA dependant RNA polymerase (RdRp) of non-segmented negative viruses as morbilliviruses uses the GP to synthesize the messengers RNA and later full-length genome copies are made via replication intermediate strands of the genome from both GP and AGP (Fig. 1a). Therefore changes in these regions may have a significant effect on the rate of transcription of viral mRNA or full-length genome replication (Mioulet *et al.,* 2001). As a first step for looking into the molecular basis of PPR pathogenesis, we decided to compare the GP and AGP of different PPRV strains. We started by determining the leader and trailer sequences of the PPRV vaccine strain. As demonstrated for the Sendai virus (SeV) (Tapparel *et al*., 1998, Vuillermoz and Roux, 2001), the promoters of morbilliviruses are bipartite and are composed of the leader plus the untranslated 5' end of the N gene for the GP, the 3' untranslated gene of the L gene plus the trailer for AGP (Fig. 1b). In Fig. 2, is presented the alignment of the different morbillivirus promoter sequences. It can be seen that while the 3' ends of the genomes are completely uniform in size, they

are two gaps in the alignment of morbillivirus AGP alignment. Indeed, for CDV and PDV, one nucleotide is missing in the 3' end of the L gene and is compensated by the presence of an extra nucleotide in the trailer making the figures for both viruses of 71 and 38 nucleotides, respectively, instead of 72 and 37 for the other morbilliviruses. Except this minor difference, the sequence alignment generated for GP and AGP of the *Morbillivirus* genus revealed a high degree of conservation. For example, the first four nucleotides of the 3' terminus, UGGU (Fig 1b), are shared between all viruses. It is believed that these four nucleotides contain the landing site for the viral RdRp. However, if the intergenic sequence is conserved among all morbilliviruses, being GAA, the intergenic L-trailer sequence is more variable and is GAU for PPRV, GUU for CDV and marine mammal morbilliviruses. In Fig. 2, are indicated the two important regions constituting each promoter according to what was proposed by Vuillermoz and Roux (2001) for the SeV. Both GP and AGP complement each other on about the first 16 nucleotides for each virus. The promoter region I is probably involved both in the encapsidation of the genome within the helical structure made by the N protein and in the interaction between the encapsidated genome and the RNA-dependant RNA polymerase (Tapparel *et al*, 1998). The second essential element, promoter region II, is composed of a series of three hexamer motifs present at position 79-84, 85-90 and 91-96 on the GP sequence. Here, the existence of the conserved discontinuous elements has been substantiated by studies with the SeV (Tapparel *et al*., 1998) and Simian virus (Murphy and Parks, 1999) showing that the three sequences can significantly influence paramyxovirus RNA replication. In this region, conserved critical C residues are found at positions 79, 85 and 91 and conserved U residues are found at positions 83 and 89, thus forming a 3'-$(CNNNUN)_2$-CNNNNN-5' conserved hexamer motif on the GP sequence and its AGP counterpart 5'-$(GNNNAN)_2$-CNNNNN-3' (Tapparel *et al*, 1998, Walpita, 2004). This motif is playing a major role in the promoter activities and is expected to contain important signals for encapsidation and transcription. The hexamer phase of this conserved motif is though to be stacked close to the three hexamers of the promoter region I

and to expose the conserved nucleotide residues on the same face of the helical nucleocapsid to form a RNA-dependent RNA polymerase binding site (Tapparel *et al*, 1998; Mioulet *et al*, 2001). In addition to these two conserved promoter regions, a third conserved region is found at position 52-71 in GP and 59-71 on AGP.

To identify possible attenuation nucleotides in the promoters of PPRV, we have amplified and sequenced the GP and AGP from 12 virulent strains recovered from field samples and compared the data with those of the vaccine strain. This later is derived from the virulent Nigeria 75/1 strain and is known to be highly attenuated for all animals into which it has been inoculated so far (Diallo *et al*., 1989). PPRV in field samples was initially detected by a RT-PCR using *Morbillivirus* universal primers targeting a highly conserved region of the N gene (positions 790-812 and 988-1012) as reported by Couacy-Hymann *et al*. (2002). Virus specific RNA was detected in nasal and eye swabs, mouth and gum erosions (Table 3). Amplification using universal primers produced 250 bp fragments from PPRV and also from RPV. PPRV specific primers, targeting the 3' terminal of the N gene, detected 390 bp fragments but did not amplify RPV (Fig. 3). Half of the samples positive with the PPR specific primers were however negative with universal primers thus indicating that the current *Morbillivirus* universal N gene based primers have limitations in detecting all strains of PPRV at the conditions of amplification we used (Table 3). Similar results were obtained by Forsyth and Barrett (1995) who used P gene primers as *Morbillivirus* universal primers. Even though the primers were designed in a zone where the gene sequence is well conserved between different morbilliviruses, a single base change at the 3' end of a primer as a result of virus strain variation can abolish the amplification. The negative results reported by Forsyth and Barrett (1995) and by us here with primers designed in conserved target sequences indicate again the necessity of using at least two sets of primers for the diagnosis of pathogens such as RNA viruses known to have high rate of mutations. Specimens which were positive with the PPRV specific primers were then submitted to two other PCR tests to amplify both GP and AGP fragments as indicated in Materials

and Methods. The amplified products were sequenced. The sequence analysis was first done by including also the sequences from 2 RPV, 2 MV, 2 CDV and that of the original strain PPRV 75/1 from which derives our vaccine strain, all being available from Genbank. Sequence alignment of the GP (107 base pairs) of virulent strains of PPRV and the vaccine strain revealed six unique nucleotide changes at positions 5 (transition U → C), 12 (transition A → G), 26 (transversion U → A), 36 (transition U → C), 42 (transition C → U) and 81 (transition C → U) in the vaccine strain (Fig. 4). Nucleotide changes on positions 5 and 12 could not be identified in our 12 field strains because they were included in the forward primer used for GP amplification. However, positions 26, 36, 42 and 81 were all confirmed to be U, U, C, and C, respectively, thus strengthening the importance of these mutations in link with the virulent/attenuated phenotype. The original virus Nigeria 75/1 wild type (Nig75/1*) and its derived vaccine strain (63 passages on cell culture) have only 2 nt changes in GP. Their AGP sequences are identical. In Nig75/1* one nucleotide change is on position 12 (transition $A_{wt} \rightarrow G_{vac}$) in link with the virulent genotype. The other change at position 83 is C which is specific to the Nig75/1* since all other PPR strains and other morbilliviruses have a U residue at this position. Preliminary results of experimental works on live animals indicated that, although virulent, PPR Nigeria 75/1 wild type has a much lower virulence than other PPR strains (Couacy-Hymann, personal communication). This observation would be in agreement with the conservation of promoter sequences between this low virulent strain and its attenuated progeny. It also suggests that other genetic determinants for PPRV virulence are probably present outside the viral promoters as already shown for RPV (Banyard *et al*, 2005). Non-segmented negative strand viruses have sequence elements at their genomic RNA ends which complement to each other (see Fig. 2 for morbillivirus 5' and 3' ends). For the SeV, it was demonstrated that the trailer is a stronger promoter than the leader and that all the 31 first nucleotides are important (Calain and Roux, 1995; Tapparel and Roux, 1996). Since the trailer serves exclusively for the replication to produce negative sense genome RNA while the leader

directs both transcription and replication, it can be assumed that the minimum sequence shared by the virus genome termini is the most important for the replication. Therefore, sequences downstream the shared area may be important for the transcription as suggested by Li and Pattnaik (1999) for vesicular stomatitis virus (VSV). The nucleotide substitutions occurring during cell culture passages for vaccine derivation can be located in any part of the genome. Those favouring specific modifications of the promoters can affect either the replication or mRNA transcription or both functions. Scanning mutagenesis analysis of the 3' end of the genome of RPV carried out by Mioulet *et al*. (2001) did not distinguish the two functions since it was based on the measurement of the reporter CAT protein expression by the minigenome construct. This study identified nucleotides 1, 3, 4, 10 and 19 as critical for the expression of the CAT protein. None of these nucleotides is concerned by the changes identified between the PPRV virulent strains and the vaccine in the work reported here. The same authors reported also the identification of residues in position 23-26 as critical. Precisely, for PPRV, RPV, MV and CDV, there is a pyrimidine → purine transversion at position 26 from the wild types (U or C) to the vaccine strains (A) (Fig. 4). Nucleotide 26 is downstream the sequence of the leader which complement with that of the trailer and it may be important for the transcription function of the leader promoter in its interaction with the RdRp or a cellular protein. It is interesting to note that for the SeV, two transversions U→ A at positions 20 and 24 in the leader sequence, outside the zone identified as critical for the replication/transcription of the viral genome, contribute to attenuate the virus for mice (Fuji *et al*., 2002). The transversion identified at the position 26 in the leader sequence of PPRV, RPV, MV and CDV may be one of the essential elements involved in the attenuation of morbilliviruses. As it can be seen in Fig. 4, there are some other nucleotide changes in the promoters sequences between virulent and vaccine strains of PPRV. The transition (C → U) at position 42 was only observed with PPRV. However, MV had a transversion (U → G) at that position and for this virus it is believed that position 26 and 42 pyrimidine-to-purine transversions belong to a nucleotide stretch between nt 17 and 42 that could

serve as contact site for a modified polymerase complex used for mRNA synthesis (Parks *et al.,* 2001). For PPRV, the conservation of a pyrimidine residue during the base 42 substitution limits probably the potential impact on the level of mRNA synthesis but may still contribute to an attenuated phenotype. Transitions at positions 5 and 12 were only identified in both PPRV and RPV. They occur within the highly conserved complementary hexamer motifs of GP and AGP starts. They affect residues identified as not critical by the scanning mutagenesis study of the RPV promoter but their change can reduce the reporter molecule production by about 60%. (Mioulet *et al*. 2001). Thus the transition at positions 5 and 12 may contribute also to the attenuated phenotype of the vaccine strain. The two other transitions of nucleotides 36 and 81 were only observed with PPRV. The pyrimidine U at position 36 of all PPRV virulent strains is unique among all other wild type and vaccine strains of morbilliviruses for which a conserved pyrimidine C is systematically found. Therefore, the transition U → C at that position gives the PPRV vaccine strain the morbillivirus conserved residue. Moreover, block mutation around that nucleotide has no effect in the promoter function of the RPV leader sequence (Mioulet *et al*., 2001). Also there is no evidence that the change in the position 81 has an impact on the PPRV virulence.

In the AGP sequence (109 bp), there was only one unique nucleotide mutation specific to the vaccine strain at position 15842, 107 nucleotides from the 5'terminus of the genome (Fig. 4). The nucleotide in that position is variable although nucleotide G is found only in the vaccine strain.

As demonstrated by Banyard *et al*. (2005) in the case of RPV, the virus genome promoters play a role in the pathogenicity of morbilliviruses. The 6 nucleotides changes observed at positions generally well conserved in the PPRV GP are likely attenuation candidates. With the advance of the reverse technology, it will be possible to generate strains with each individual mutation or group of mutations and evaluate their impact on the virus virulence. Among all the candidates, the most interesting is certainly the mutation observed at the position 26 because not only it is found in the genome of RPV, PPRV, MV and CDV vaccines strains but also it is in the area identified as

involved in the pathogenesis of SeV, another paramyxovirus (Fuji *et al.*, 2002). Likely, any mutation in the promoters involved in the attenuation of PPRV may play in concert with other critical changes not only within the promoters but also in other virus genes as demonstrated with RPV. Indeed, as demonstrated Banyard *et al.,* (2005) and Baron *et al.* (2005) for RPV, the attenuation of morbillivirus may be a result of changes in different genes of the genome.

Differences in virulence of PPR between goats and sheep reported in Africa were not supported by our sequence analysis of PPRV promoters. Also, specimen type did not play any role as there was 100% homology between sequences derived from nasal swabs, lung and lymph nodes from the same goat or sheep (data not shown). In contrast, within a same geographic region like Israel, some nucleotide changes may occur in a short period. Interestingly, mutations were observed between 1999 and 2000 in both GP and AGP sequences of goat as well as sheep strains, while the sequences are conserved after 2000 (data not shown). None of these viruses had been passaged on cell culture, which could have introduced genetic variability. It is however not clear what triggered these nucleotide changes.

Use of the GP and AGP sequences of PPRV strains to determine the phylogenetic relationship

Despite the overall high conservation of PPRV promoters, some variations were seen in parts of their sequence (Fig. 3). Phylogenetic analysis of tandemly linked GP and AGP (Fig. 5a) of PPR field strains from different areas and of the vaccine strain (Nig 75 vac) showed that these variations could lead to a geographic clustering of the strains. Earlier, Shaila *et al*. (1996), and then Dhar *et al.* (2002) demonstrated that isolates of PPRV could be grouped into four distinct lineages on the basis of partial sequence analysis of the fusion (F) protein gene. Relationship deduced from a method based on the principle of parsimony established that the promoter sequences were also lineage specific. Similar lineage specific base changes in the GP and AGP were also observed with rinderpest viruses (Banyard *et al.,* 2005). This illustrates the possibility of using very short sequence

regions at the end of the virus genome for molecular typing of isolates, both for virulence and lineage discrimination. Lineage speciation was identical when using independently the GP or AGP sequences (data not shown). However, combination of the two allows to increase the bootstrap values and to strengthen the molecular typing of PPRV isolates next to other morbilliviruses. Furthermore, phylogenetic analysis of the GP-AGP tandem of *Paramyxoviridae* listed in Table 2, confirms that morbilliviruses form a unique cluster within the family (Fig. 5b). Although having unique features that clearly distinguish them from other paramyxoviruses, Henipa and Tupaia viruses are significantly closer to morbilliviruses than to any other virus genera (bootstrap values higher than 80%). The same observation was obtained when grouping the viruses through full-genome sequences (Lwamba *et al*, 2005). The closer relationship observed among the genomic termini of these viruses could be supported by the slightly better identity of the N gene between Hendra and *Morbillivirus* (Yu *et al., 1998*) and of the L gene between *Henipa*, *Tupaia* and *Morbillivirus* (Harcourt *et al, 2001*). In particular, the polymerase contains a multifunctional G-rich motif in the domain VI that is identical in all these virsues. This phylogenetic proximity observed by independent sequence analysis of the viral promoters and the N and L proteins, may result from the physical constraints applied to the viral ribonucleoprotein (RNP) in which these three components have to be intimately linked to form a functional complex. Thus, one can imagine that co-evolution of these interacting molecules is necessary to maintain a functional RNP and a viable virus. Although the bootstrapping was not significant, further *Paramyxoviridae* discrimination was still possible and in agreement with the classification obtained using the full-genomes (Lwamba *et al*, 2005). The low bootstrap values are resulting from the promoter sequence shortness used for molecular discrimination.

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Table 1. Samples processed for RT-PCR and further genome and antigenome promoters sequence analysis

No.	Identification	Sequence coding	Country of origin	Animal species	Specimen	Cell culture, cell type and passage no.
1	Ethiopia-1994	Ethio/94	Ethiopia	Goat		Sheep fibroblast-3
2	Nigeria75/1wildtype	Nig75wt				Vero-1
3	RP-RBOKvac-1986*	-	UK, Pirbright			RBT-1
4	Israel-1998	Isrl/98	Israel	Goat	lung	Not passaged
5	Israel-1999	Isrl/99	Israel	Sheep	eye swab	«
6	Israel-2000a**	Isrl/00a	Israel	Goat	lung	«
7	Israel-2000b	-	Israel	Goat	lung	«
8	Israel-2000c	Isrl/00c	Israel	Sheep	eye swab	«
9	Israel-2000d	-	Israel	Sheep	lung	«
10	Israel-2000e	-	Israel	Sheep	eye swab	«
11	Israel-2000f	-	Israel	Sheep	intestine	«
12	Israel-2000g	-	Israel	Sheep	eye swab	«
13	Israel-2001a	Irsl/01a	Israel	Sheep	spleen	«
14	Israel-2001b	Isrl/01b	Israel	Sheep	lung	«
15	Israel-2001c	-	Israel	Sheep	lung	«
16	Israel-2001d	-	Israel	Goat	lung	«
17	Israel-2001e	-	Israel	Sheep	lymph node	«
18	Israel-2001f	-	Israel	Goat	lung	«
19	Israel-2002	-	Israel	Sheep	eye swab	«
20	Israel-2003a	Isrl/03a	Israel	Sheep	eye swab	«
21	Israel-2003b	-	Israel	Sheep	eye swab	«
22	Israel-2003c	-	Israel	Sheep	eye swab	«
23	Israel-2003d	-	Israel	Sheep	lung	«
24	Cote d'Ivoire-1989	C'Ivo/89	Cote d'Ivoire	goat-	lung-	Sheep-fibroblast-3
25	India-Calcutta-1995	Ind-C/95	India, Calcutta	Goat	lymph node	Sheep-fibroblast26
26	India-Pradesh-1995	Ind-P/95	India, Pradesh	Goat	lung	Sheep-kidney15
27	Tadjikistan-2005	Tadijk/05	Tadjikistan	-	-	-

* The rinderpest virus strain (RP-RBOKvac) used for RT-PCR control in this study was not sequenced (the sequence for this virus was collected from GenBank, see table 2).

** letters at the end of the year indicate different localities of specimen collection.

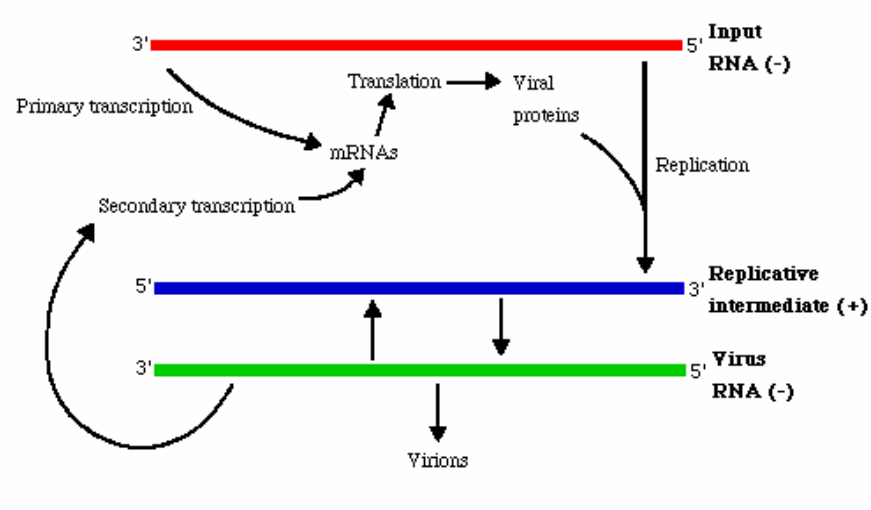
Table 2. Selected strains of *Paramyxoviridae* from Gene bank database for phylogenetic analysis of genome and antigenome promoter sequences

No.	Virus	Sequence coding	Accession No.	Author	Publication
1	Nigeria75/1vac	Nig75vac	X74443	Berhé *et al*, 2005	NCBI
2	PPRV Nigeria 75/1 (wild type)	Nig75/1*	AJ879463 AJ879467	Dash and Barrett, 2005	NCBI
3	PPRV Nigeria76/1 (wild type)	Nig76/1	AJ879466 AJ879468	Dash and Barrett, 2005	NCBI
4	PPRV Oman-83	Oman/83	AJ879464 AJ879469	Dash and Barrett, 2005	NCBI
5	PPRV GuineaB-91	Guin-B/91	AJ879465 AJ879470	Dash and Barrett, 2005	NCBI
6	PPRV Turkey-2000	Turk/00	NC-006383	Bailey *et al*., 2005	Virus Res.
7	Rinderpest virus	RPV	NC-006296	Baron *et al*., 1996	J.Gen.Virol.
8	Measles virus	MV	NC-001498	Takeuchi *et al*., 2000	Virus Genes
9	Dolphin morbillivirus	DMV	NC-005283	Rima *et al*., 2003	NCBI
10	Bovine respiratory syncitial virus	BRSV	NC-001989	Buchholz *et al*., 1999	J. Virol.
11	Bovine parainfluenza virus	BPIV	NC-002161	Bailly *et al*., 2000	Virus Genes
12	Human respiratory syncitial virus	HRSV	NC-001781	Karron *et al*., 1997	Proc.Natl.Acad. Sci. USA
13	Human parainfluenza virus	HPIV	NC-003461	Newman *et al*., 2002	Virus Genes
14	Newcastle disease virus	NDV	NC-002617	Sellers and Seal, 2000	NCBI
15	Avian paramyxovirus	APMV	NC-003043	Chang *et al*., 2001	NCBI
16	Sendai virus	Sendai	NC-001552	Itoh *et al*., 1997	J.Gen.Virol.
17	Mumps virus	Mumps	NC-002200	Okazaki *et al*., 1992	Virology
18	Nipah virus	Nipah	NC-002728	Harcourt *et al*., 2001	Virology
19	Hendra virus	Hendra	NC-001906	Wang *et al*., 2000	J.Virol.
20	Tupaia virus	Tupaia	NC-002199	Tidona *et al*., 1999	Virology

Table 3. Results of RT-PCR using target gene specific primers. All negative results were confirmed by repeated tests

No.	Identification	Morbillivirus specific PCR	PPR specific PCR	Genome promoter specific PCR	Antigenome promoter specific PCR
1	Nigeria75/1vac	+	+	+	+
2	Nigeria75/1 wt	+	+	+	+
3	Ethiopia-1994	+	+	+	+
4	RP-RBOKvac-1986	+	-	-	-
5	Israel-1998	+	+	+	+
6	Israel-1999	-	+	+	+
7	Israel-2000a	-	+	+	+
8	Israel-2000b	-	+	+	+
9	Israel-2000c	-	+	+	+
10	Israel-2000d	-	+	+	+
11	Israel-2001a	-	+	+	+
12	Israel-2001b	-	+	+	+
13	Israel-2003a	+	+	+	+
14	Israel-2003b	+	+	+	+
15	Israel-2003c	-	+	+	+

(a)



(b)

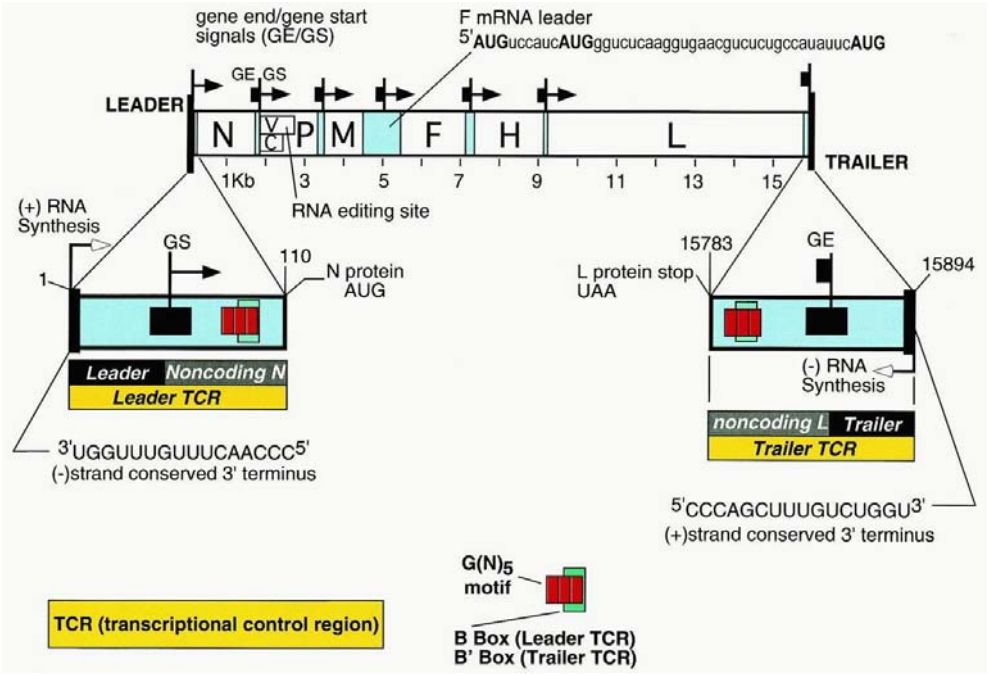
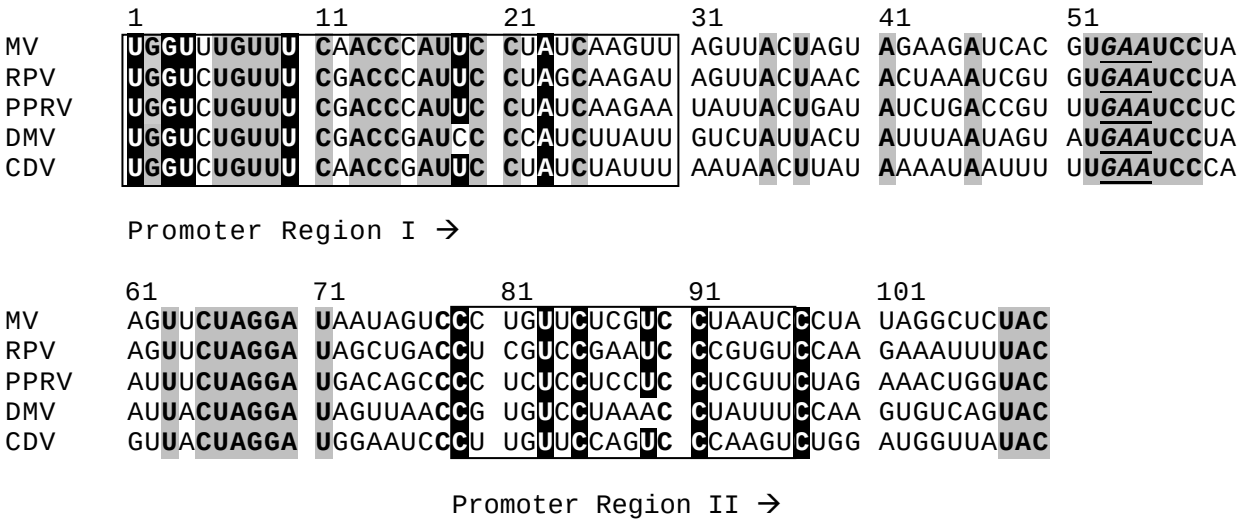


Fig. 1 *Morbillivirus* genome organisation. (a) *Morbillivirus* replication pathway. Full-length genome copies are made via replication intermediate strands of the minigenome. (b) Genes and promoters of *Morbillivirus* (from Parks *et al*, 2001): the protein coding regions (N, P, V, C, M, F, H, and L), noncoding intergenic regions and the leader and trailer regions along with specialized sequence motifs are shown. The genome promoter includes the leader sequence and the non coding regions N at the 3' end of the genomic RNA. The antigenome promoter includes the trailer sequence and the untranslated regions of the L gene at 5' end. Gene start (GS) and gene end (GE), enclosing the intergenic trinucleotide motifs are also shown.

(a)



(b)

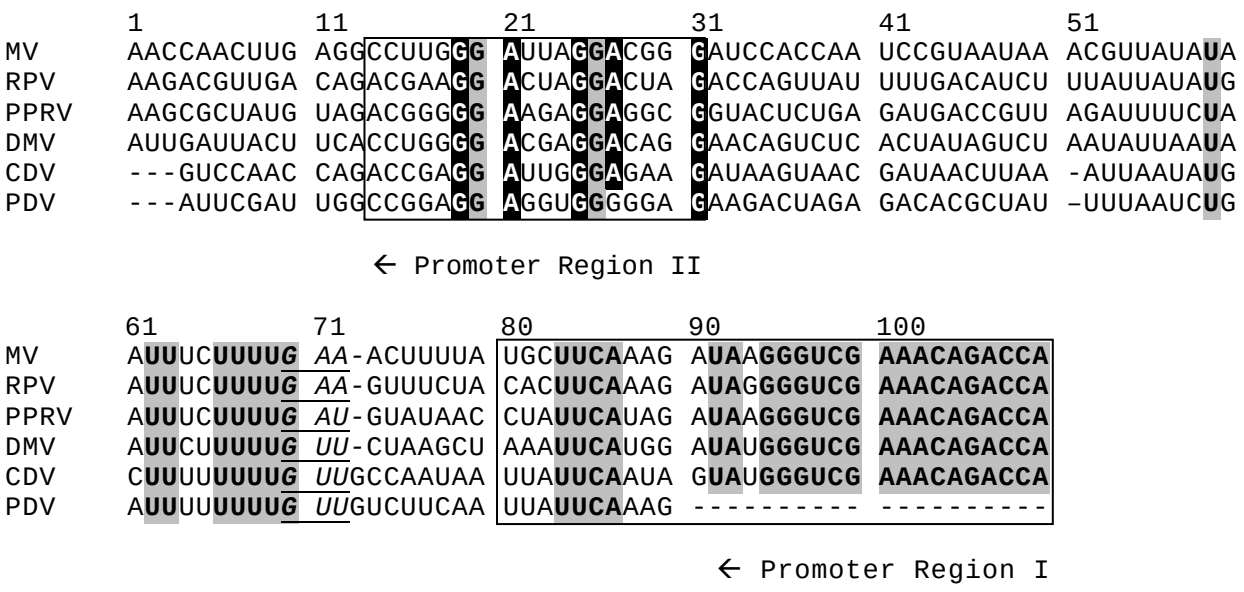


Fig. 2 Alignment of the genomic (a) and antigenomic (b) promoters of the members of the genus *Morbillivirus*. The boxes delineate the promoter region I, that includes the first 30 nucleotides and the promoter region II composed of the three sequential hexamer residues. Bold letters in grey boxes represent identical residues. The trinucleotide inter-cistronic sequences are underlined, while dashes represent gaps introduced during alignment. Underscores mean no sequence available for that position. Bold white letters in black boxes represent the critical residues that neutralise the GP promoter activity of RPV (Mioulet *et al*, 2001) and the AGP promoter activity of MV (Walpita, P, 2004). Accession numbers for this figure are CDV (AY466011), DMV (NC_005283), RPV (Z30697), MV (K01711), PPRV (X74443).

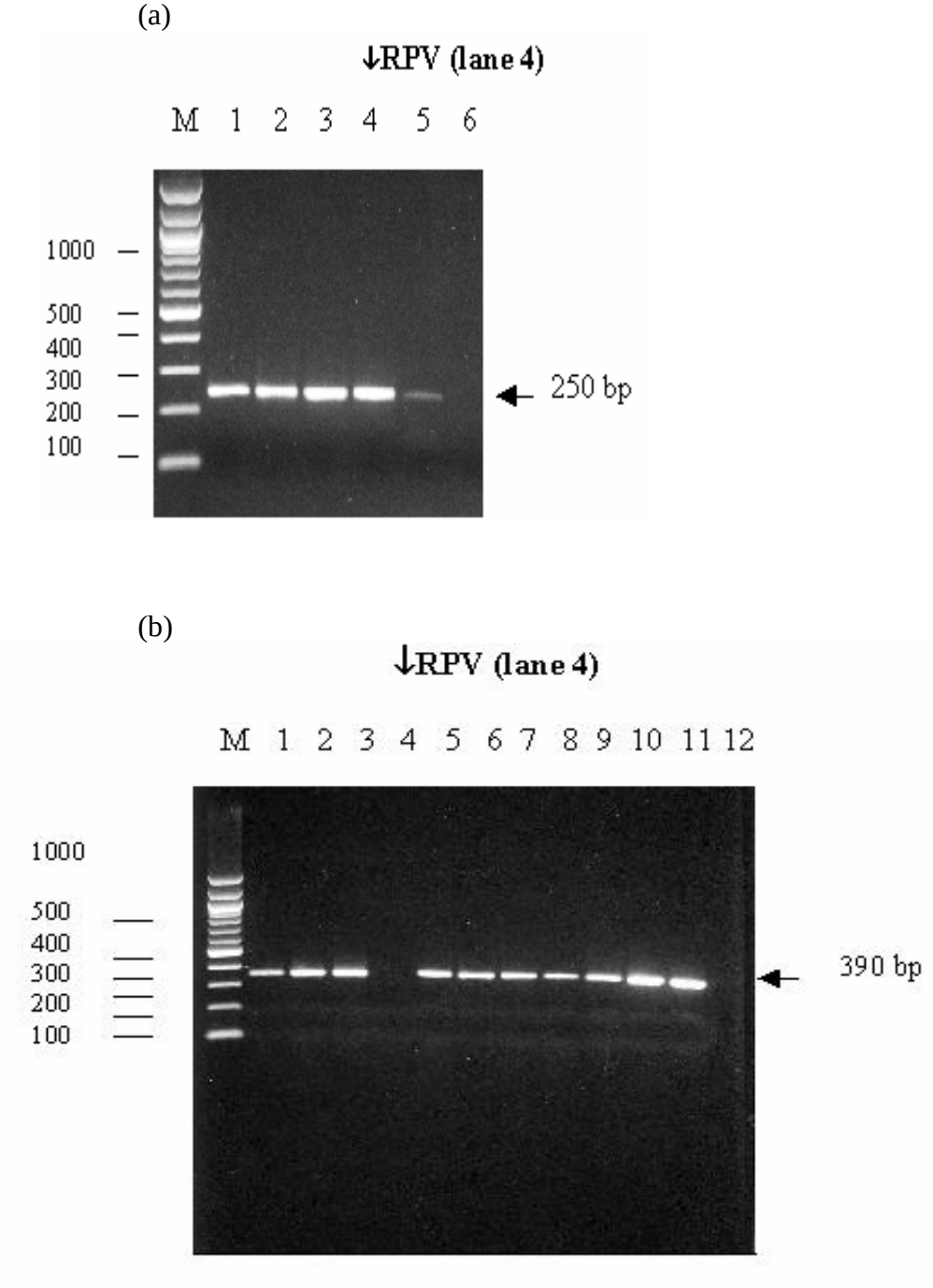
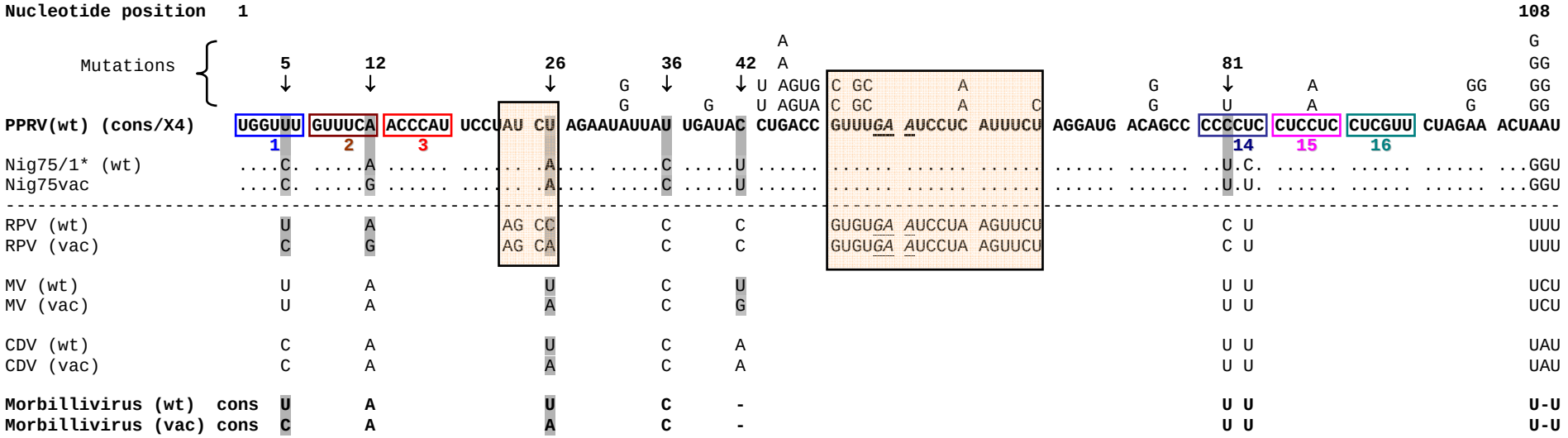


Fig. 3 Agarose gel electrophoresis of PCR products. **(a)**. amplification was carried out using universal primers; Lane 4 is RPV and others were PPRV strains: lane 6 is a negative control **(b)**. amplification was carried out using PPR specific primers. Lane 4 is RPV and others were PPRV strains; lane 12 in 3B is a negative control. **M.** represents 100bp DNA ladder (Biolabs, USA).

(a)


Nucleotide position 1
108
Mutations
5
12
26
36
42
81
PPRV(wt) (cons/X4)
UGGUUU GUUUCA ACCCAU UCCUAU CU AGAAUAUUAU UGAUAC CUGACC GUUUGA AUCCUC AUUUCU AGGAUG ACAGCC CCCCUC CUCCUC CUCGUU CUAGAA ACUAAU
1 2 3 14 15 16
Nig75/1* (wt)
Nig75vac
RPV (wt)
RPV (vac)
MV (wt)
MV (vac)
CDV (wt)
CDV (vac)
Morbillivirus (wt) cons
Morbillivirus (vac) cons

(b)

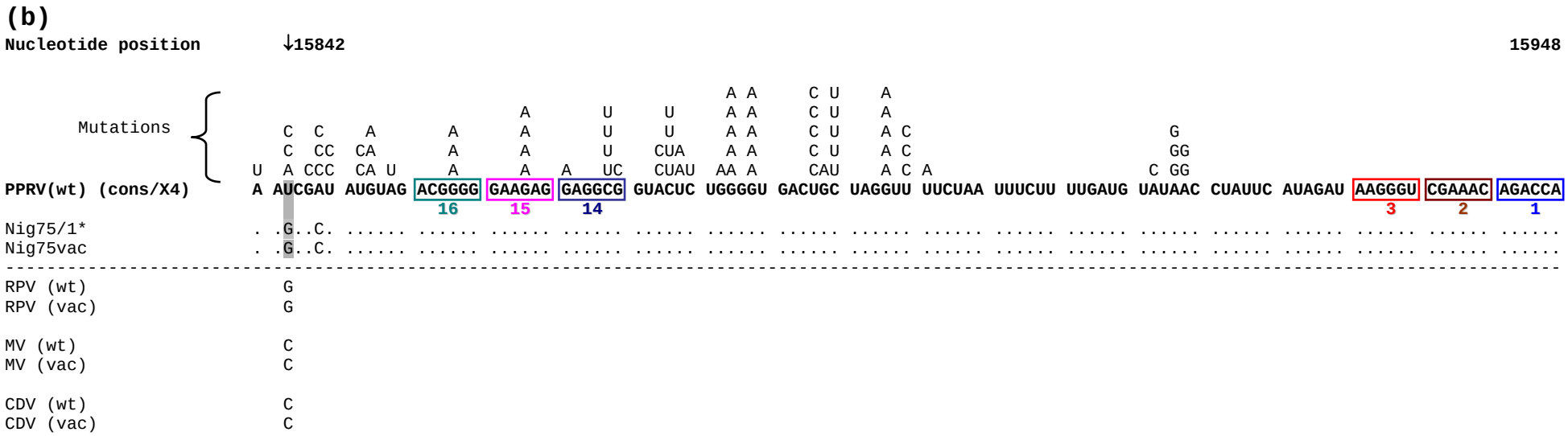
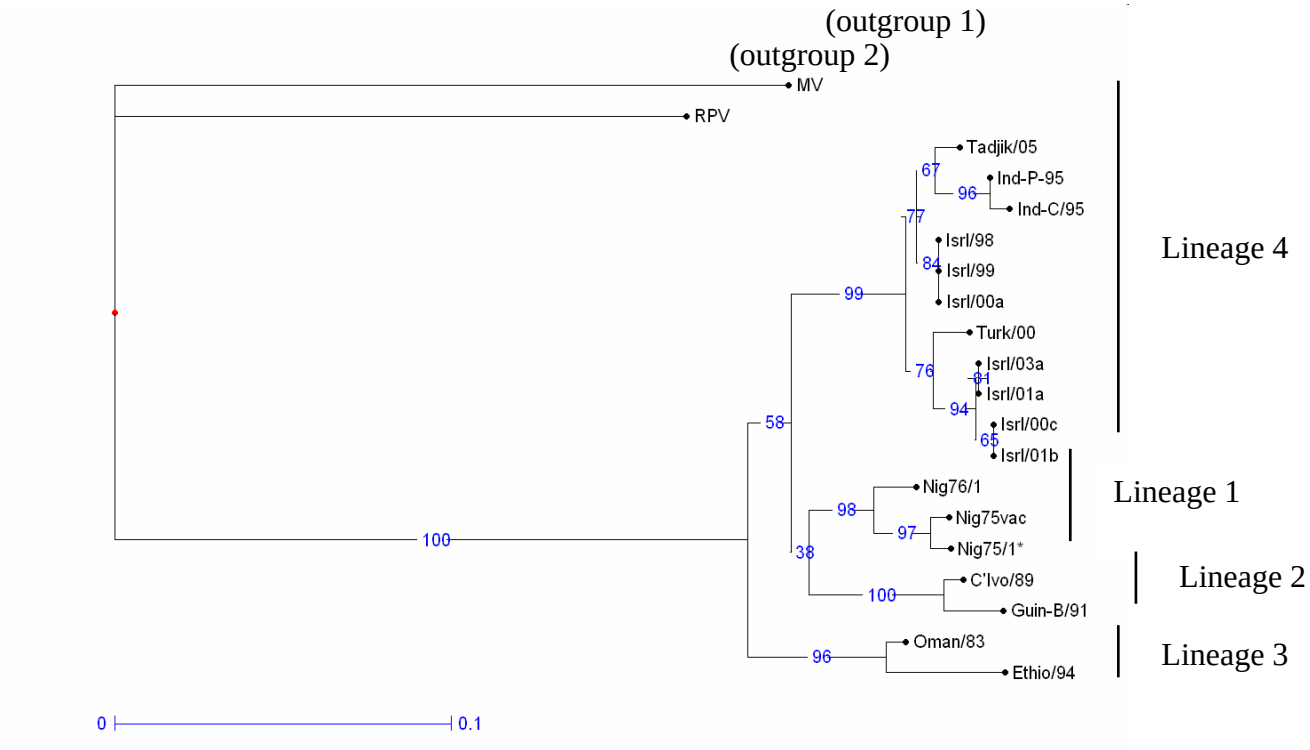

Nucleotide position ↓15842
15948
Mutations
PPRV(wt) (cons/X4)
A AUCGAU AUGUAG ACGGGG GAAGAG GAGGCG GUACUC UGGGGU GACUGC UAGGUU UUCUAA UUUCUU UUGAUG UAUAAC CUAUUC AUAGAU AAGGGU CGAAAC AGACCA
16 15 14 3 2 1
Nig75/1*
Nig75vac
RPV (wt)
RPV (vac)
MV (wt)
MV (vac)
CDV (wt)
CDV (vac)

Fig. 4 Analysis of GP (a) and AGP (b) promoter sequences of PPRV strains and comparison with other morbilliviruses on some interesting positions (shown by number and vertical arrows). Consensus sequences of wild type PPRV are shown as nucleotide hexamers from the ends of GP and AGP, respectively. Above the consensus, are shown the nature and frequency of mutations that were found. Directly below the consensus, the sequence of the vaccine and wild type strain of PPRV Nigeria 75 are aligned (dots mean conservative residue compared to consensus sequence). Below the dash line, the nucleotides of RPV, MV and CDV morbilliviruses at the interesting positions are reported. For GP, consensus nucleotides at the interesting positions for vaccine and wild type strains of morbilliviruses are also indicated. Positions where vaccine and wild type strains differ are emphasized by grey boxes. Pale orange Boxes indicated the blocks found to be critic on RPV by Mioulet *et al* (2001). Coloured hexamers represent the GP and AGP promoter regions I and II that are essential for transcription and replication activity (Tapparel *et al*, 1998). Accession numbers used in this figure are PPRV (Table 2), RPV (vac: Z30697, wt: Z33635, AY775545), MV (vac: AF266286, AF266289, wt: AF266288, AB012948), CDV (vac: AF305419, AF378705, wt: AF164967, X74443

(a)


(outgroup 1)
(outgroup 2)
MV
RPV
Tadjik/05
Ind-P-95
Ind-C/95
Isrl/98
Isrl/99
Isrl/00a
Turk/00
Isrl/03a
Isrl/01a
Isrl/00c
Isrl/01b
Nig76/1
Nig75vac
Nig75/1*
C'Ivo/89
Guin-B/91
Oman/83
Ethio/94
Lineage 4
Lineage 1
Lineage 2
Lineage 3
67
96
77
84
99
76
94
65
58
98
97
38
100
100
96
100
0
0.1

(b)

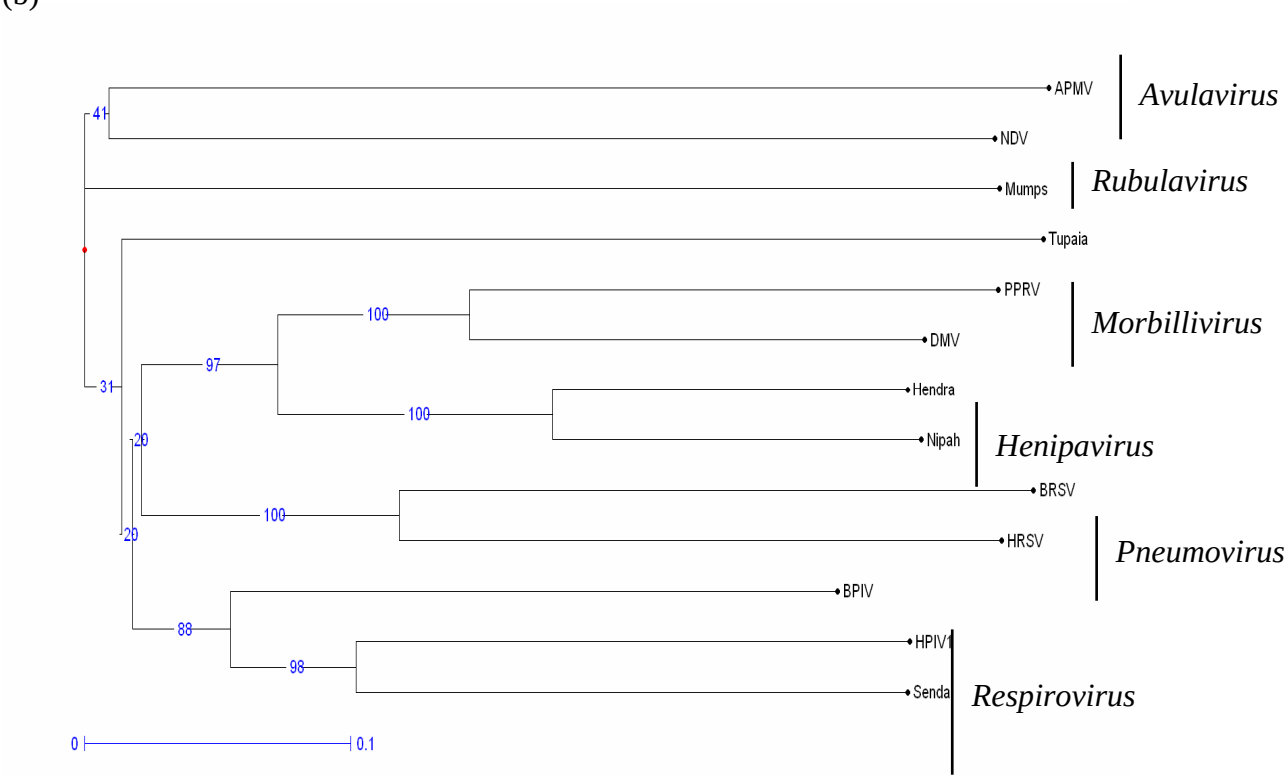

APMV
NDV
Mumps
Tupaia
PPRV
DMV
Hendra
Nipah
BRSV
HRSV
BPIV
HPIV1
Senda
Avulavirus
Rubulavirus
Morbillivirus
Henipavirus
Pneumovirus
Respirovirus
41
31
20
20
97
100
100
100
88
98
0
0.1

Fig. 5 Phylogenetic analysis of the genome and antigenome promoters, combined in tandem sequences. (a) Promoter sequences of PPR field and vaccine (Nig75vac) strains with two outgroups (MV and RPV) added in order to root the tree. The tree was constructed using maximum parsimony (Neighbor joining method, Saitou and Nei, 1987) following a CLUSTAL W alignment of the nucleotide sequences. The numbers indicate the bootstrap values after 1000 resamplings. Scale represents the degree of similarity. PPRV lineages were placed according to Shaila *et al* (1996). In the tree (b), the same procedure was applied on the corresponding promoter sequences of various *Paramyxoviridae*. All strains used in this analysis are described in Table 1 and 2, except PPRV, which is the strain Nig75vac.

Chapter 6

General discussion

6.1. Epidemiology of PPR in Ethiopia

The observations made on pathogenicity traits of PPRV in the natural biological life cycle indicate that it is a rather complex and multifaceted phenomenon for such recently classified virus. Even though it is known to be a single viral strain in the *Morbillivirus* genus and closely related to the other five members of the group with their restricted host range specificity, PPR infects most ruminant animals with varying degree of clinical and pathological outcome. Both large and small ruminants are readily infected and there is efficient natural transmission in the field in the large ruminant population from infected small ruminants as detected by seroconversion. However, infections in this population are generally asymptomatic and cannot be transmitted to contact animals, as confirmed by animal experimentation. Determinant factors for difference in PPRV pathogenicity according to the concerned species are not yet known. The figure is even more complicated in goats and sheep because in certain regions of Africa, some goat breeds may have subclinical infection while others manifest severe clinical disease and death. In East Africa (Ethiopia), PPR caused high mortality in goats, while sheep in contact had not been affected (Roeder *et al*. 1994). In Asia and the Middle East both goats and sheep are evenly affected and the virus caused high mortality in both species of animals (Shaila *et al*., 1989). Pattern of disease incidence and severity depends on the immunity level in the population, duration of maternal antibody, occurrence of other bacterial or parasitic diseases, movement and gathering of large populations for grazing or marketing purposes, feed stress and seasonal and climatic changes. To clarify the situation prevailing in Ethiopia, a field observation was conducted. Livestock production is an integral part of the country's agricultural system. The different ecological zones allow the production of various species of livestock which represent a major national

resource. Small scale and pastoralist farming systems in Ethiopia constitute reserves for family emergency needs and food security. In addition, animal products give major export commodities of about 50 millions USD annually. Sheep and goats represent more than 30% of the domestic meat consumption. Pastoralists and semi-pastoralists sustain their culture, life style and pride on their livestock. Animal diseases affect productivity by 50 to 60% a year by reducing production potentials of the indigenous stock and restricting the introduction of more productive exotic breeds. PPR was confirmed in 1991 and outbreaks were seen in goats while sheep in the same area were not affected. In the present work, we showed that the antibody seroprevalence in camel, cattle and sheep confirmed natural transmission of infection in these animals without clinical disease. The level of virus circulation in sheep was similar to the one observed in goats which were suffering from the disease. The apparent absence of pathogenicity in sheep could result from a particular resistance of the local species and/or a loss of virulence of the Ethiopian PPRV strains for sheep. Low virulence outcome may result from a reduced capacity of the virus to infect cells or to replicate in these cells, resulting in a lower viral antigen distribution through different organs and tissues. Such characteristics may account for the milder clinical disease and lower mortality. Therefore, differences in virulence can be related to the virus load received by the animals, the efficiency of intercellular spreading inside the body and capacity to induce damages in infected cells. However, other factors which determine strain virulence remain essentially unknown and those identified so far have not been related to a single event. The capability of cells to be infected and support active virus replication has important implications on the pathogenesis and epidemiology of the disease. Therefore, we were interested to test the cell susceptibility of PPRV.

6.2. Monitoring of PPRV virulence *in vitro* in cell culture systems

In light of the epidemiological observation in the field, where PPRV expressed different degrees of pathogenicity in different species of animals. Monitoring of PPRV *in vitro,* in cell culture systems was conducted to determine if active replication of PPR and rinderpest viruses occurred in the same cell lines and thus if virus antigen expression can vary according to the cell type. The relative capacity of the cell to support active replication of PPRV and rinderpest virus (RPV) was determined by flow cytometry analysis. This analysis used labelling with MAbs against N and H proteins. Thus, the use of these virus specific monoclonal antibodies enabled to detect differences in cell susceptibility of PPRV compared to RPV. Vero and 293T cells supported productive infection by both viruses. However, comparison of the infections showed that the kinetics of replication in Vero and 293T cells was different in terms of virus antigen expression. The production of virus antigen and infectious virus progeny at the earlier time points post infection was lower in infected Vero cells compared to 293T cells. These results suggested that replication in 293T cells was initially more efficient. However, at later stages during the infectious cycle, the infection of Vero cells became more productive. The reason for the early efficient infection of 293T cells compared to Vero may relate to the difference levels of cytoplasmic, particularly lysosomal, activity in the cell types or to differences in endocytic and phagocytic activities. Nevertheless, these differences did not prevent the virus from eventual spreading to all cells at 120 or 144 hours post infection. MOCL5 cells did not support replication of PPRV and RPV (data not shown). Although of ovine origin, these cells may lack a specific attachment protein or an intracellular host protein necessary for efficient replication. Our results showed that B95a marmoset lymphoblastoid cells supported RPV replication, but they did not sustain replication of PPRV. Further attempts to adapt the PPRV to B95a cell line by six blind serial passages failed. This is contradictory to the report of (Das *et al*., 2000) in which PPRV could be adapted to B95a cells

after five to six blind passages. Nonetheless, PPRV seems to behave differently from all morbilliviruses tested so far. RPV (Kobune *et al*., 1991), MV (Kobune *et al*., 1990) and canine distemper virus (Seki *et al*., 2003) replicate and can be isolated successfully in B95a cell line. It may be that these cells lack an efficient receptor for the attachment H hemagglutinin protein of PPRV or are deficient for an essential intracellular host protein necessary for efficient PPRV replication. Cellular receptors are one of the major determinants of the host range and tissue tropism of a virus (Wild *et al*., 1991, Tatsuo *et al*., 2001). MV uses CD46 receptor for Vero but SLAM for B95a (Takeuchui *et al*., 2002). The H (attachment) glycoproteins of morbilliviruses are divergent and this variation may play a role in host cell specificity (Blixenkrone-Müller *et al*., 1996). Therefore, we carried out an amino-acid sequence analysis of the H protein of several cell-adapted vaccine strains and wild type strains of PPRV, RPV, measles virus (MV) and canine distemper virus (CDV). We observed that all critical residues identified so far on the H-MV protein for the interaction with the SLAM (CD150 receptor) were conserved in the H-PPRV. However, we could identify 27 unique mutations on the H-PPRV at conserved positions for MV, RPV and CDV. In order to anticipate the potential effect of these mutations on the H structure and function, we decided to use the hypothetical model of Masse *et al* (2004) established on the H-MV on the H amino-acid sequence of our H-PPRV. Surface modelling using this hypothetical 3D structural model confirmed one mutation as critical for interaction with the SLAM of B95a cells (Masse *et al*, 2004). Thus, the hydrophobic non polar aliphatic residue V525 of H-MV was replaced by the hydrophobic non polar aliphatic I525. The model also detected two conformational differences between the H-MV and H-PPRV molecules. First, on top side, the surface of critical residues was slightly modified, may be as a consequence of the mutation V508 → I508. Second, the critical residue L526 on H-MV was replaced by amino-acids Y540 and I542. Whether these modifications may account for a defective interaction of the H-PPRV

with the B95a SLAM receptor will need further analysis, as the non-susceptibility of B95a cells to PPRV cannot be explained simply by sequence modification of the H protein.

6.3. Sequence analysis of PPRV and other morbilliviruses

Molecular basis for attenuation of the virus vaccine strain was sought with comparative sequence analysis of the genome and antigenome promoters (GP and AGP). Thus, GP and AGP of the peste des petits ruminants (PPR) virus, amplified from different pathological samples of sheep and goat origins, were sequenced and compared with corresponding sequences of PPR vaccine strain and other morbilliviruses. The promoters of morbilliviruses are composed of the leader plus the untranslated 3' end of the N gene for the GP, the 5' untranslated gene of the L gene plus the trailer for AGP. Both GP and AGP complement each other on about the first 16 nucleotides for each virus. The promoter region I is probably involved both in the encapsidation of the genome within the helical structure made by the N protein and in the interaction between the encapsidated genome and the RNA-dependant RNA polymerase (Tapparel *et al*, 1998). The conserved motif is thought to be attached to the three hexamers of the promoter region I of the helical nucleocapsid to form the polymerase binding site (Tapparel *et al*, 1998; Mioulet *et al*, 2001). The second essential element, promoter region II, is composed of a series of three hexamer motifs present at position 79-84, 85-90 and 91-96 on the GP sequence. In addition to these two conserved promoter regions, a third conserved region is found at position 52-71 in GP and 59-71 on AGP. The sequence alignment generated for GP and AGP of the *Morbillivirus* genus revealed a high degree of conservation, and so, the first four nucleotides of the 3' terminus, UGGU, are shared between all viruses. It is believed that these four nucleotides contain the landing site for the viral RNA dependent RNA polymerase. In the cycle of infection, the RNA dependant RNA polymerase of non-segmented negative viruses as morbilliviruses uses the GP to synthesize the messengers RNA and later full-length genome copies are made via replication intermediate strands of the genome from

both GP and AGP. Therefore, changes in these regions may have a significant effect on the rate of transcription of viral mRNA or full-length genome replication (Mioulet *et al.,* 2001).

To identify possible attenuation nucleotides in the promoters of PPRV, we have amplified and sequenced the GP and AGP of 12 virulent strains recovered from field samples and compared the data with those of the vaccine strain. This later is derived from the virulent Nigeria 75/1 strain and is known to be highly attenuated for all animals into which it has been inoculated so far (Diallo *et al*., 1989b). PPRV in field samples was initially detected by a RT-PCR (Couacy-Hymann *et al*. (2002). Virus specific RNA was detected in nasal and eye swabs, mouth and gum erosions. Specimens which were positive with the PPRV specific primers were then submitted to two other PCR tests to amplify both GP and AGP fragments. The amplified products were sequenced. The sequence analysis was first done by including also the sequences from 2 RPV, 2 MV, 2 CDV and that of the original strain PPRV 75/1 from which derives our vaccine strain, all being available from Genbank. Sequence alignment of the GP (107 base pairs) of virulent strains of PPRV and the vaccine strain revealed six unique nucleotide changes at positions 5, 12, 26, 36, 42 and 81 in the vaccine strain. Nucleotide changes on positions 5 and 12 could not be identified in our 12 field strains because they were included in the forward primer used for GP amplification. However, mutations at positions 26, 36, 42 and 81 were all confirmed, thus strengthening their importance in link with the virulent/attenuated phenotype. The original virus Nigeria 75/1 wild type (Nig75/1*) and its derived vaccine strain (63 passages on cell culture) have only 2 nt changes in GP. Their AGP sequences are identical. In Nig75/1* one nucleotide change, on position 12 is related to the virulent genotype as expected, whereas the other at position 83 is specific of Nig75/1* since all other PPR strains and other morbilliviruses have the same residue at that position. Preliminary results of experimental works on live animals indicated that, although virulent, PPR Nigeria 75/1 wild type has a much lower virulence than other PPR strains (Couacy-

Hymann, personal communication). This observation would be in agreement with the conservation of promoter sequences between this low virulent strain and its attenuated progeny. Mioul*et al*. (2001) reported residues in position 23-26 as critical. Precisely, for PPRV, RPV, MV and CDV, there is a pyrimidine → purine transversion at position 26 from the wild type to the vaccine strains (A). Nucleotide 26 is downstream the sequence of the leader which complement with that of the trailer and it may be important for the transcription function of the leader promoter in its interaction with the cellular protein. The transversion identified at the position 26 in the leader sequence of PPRV, RPV, MV and CDV may be one of the essential elements involved in the attenuation of morbilliviruses. The transition at position 42 was only observed with PPRV. However, MV had a transversion at that position and for this virus it is believed that position 26 and 42 pyrimidine-to-purine transversions belong to a nucleotide stretch between nt 17 and 42 that could serve as contact site for a modified polymerase complex used for mRNA synthesis (Parks *et al.,* 2001). For PPRV, the conservation of a pyrimidine residue during the base 42 substitution limits probably the potential impact on the level of mRNA synthesis but may still contribute to an attenuated phenotype. Transitions at positions 5 and 12 were only identified in both PPRV and RPV. They occur within the highly conserved complementary hexamer motifs of GP and AGP starts. They affect residues identified as not critical by the scanning mutagenesis study of the RPV promoter but their change can reduce the reporter molecule production by about 60%. (Mioulet *et al*., 2001). Thus the transition at positions 5 and 12 may contribute also to the attenuated phenotype of the vaccine strain. The two other transitions of nucleotides 36 and 81 were only observed with PPRV. The pyrimidine at position 36 of all PPRV virulent strains is unique among all other wild type and vaccine strains of morbilliviruses for which a conserved pyrimidine is systematically found. Therefore, the transition at that position gives the PPRV vaccine strain the same morbillivirus conserved residue. Moreover, block mutation around

that nucleotide has no effect in the promoter function of the RPV leader sequence (Mioulet *et al*., 2001). Also there is no evidence that the change in the position 81 has an impact on the PPRV virulence.

In the AGP sequence (109 bp), there was only one unique nucleotide mutation specific to the vaccine strain at position 15842, 107 nucleotides from the 5'terminus of the genome (Fig. 4). The nucleotide in that position is variable although nucleotide G is found only in the vaccine strain.

As demonstrated by Banyard *et al*. (2005) in the case of RPV, the virus genome promoters play a role in the pathogenicity of morbilliviruses. The 6 nucleotides changes observed at positions generally well conserved in the PPRV GP are likely attenuation candidates. Among all the candidates, the most interesting is certainly the mutation observed at the position 26 because not only it is found in the genome of RPV, PPRV, MV and CDV vaccines strains but also it is in the area identified as involved in the pathogenesis of SeV, another paramyxovirus (Fuji *et al*., 2002). Differences in virulence of PPR between goats and sheep reported in Africa were not supported by our sequence analysis of PPRV promoters. Also, specimen type did not play any role as there was 100% homology between sequences derived from nasal swabs, lung and lymph nodes from the same goat or sheep (data not shown). In contrast, within a same geographic region like Israel, some nucleotide changes may occur in a short period. Interestingly, mutations were observed between 1999 and 2000 in both GP and AGP sequences of goat as well as sheep strains, while the sequences are conserved after 2000 (data not shown). None of these viruses had been passaged on cell culture, which could have introduced genetic variability. It is however not clear what triggered these nucleotide changes.

Phylogenetic analysis of tandemly linked GP and AGP of PPR field strains from different areas and of the vaccine strain (Nig 75 vac) showed that these variations could lead to a geographic clustering of the strains. Earlier, Shaila *et al*. (1996), and then Dhar *et al*. (2002)

demonstrated that isolates of PPRV could be grouped into four distinct lineages on the basis of partial sequence analysis of the fusion (F) protein gene. Relationship deduced from a method based on the principle of parsimony established that the promoter sequences were also lineage specific. Similar lineage specific base changes in the GP and AGP were also observed with rinderpest viruses (Banyard *et al.,* 2005). This illustrates the possibility of using very short sequence regions at the end of the virus genome for molecular typing of isolates, both for virulence and lineage discrimination. Lineage speciation was identical when using independently the GP or AGP sequences (data not shown). However, combination of the two allows to increase the bootstrap values and to strengthen the molecular typing of PPRV isolates next to other morbilliviruses. Furthermore, phylogenetic analysis of the GP-AGP tandem of *Paramyxoviridae*, confirms that morbilliviruses form a unique cluster within the family. Although having unique features that clearly distinguish them from other paramyxoviruses, Henipa and Tupaia viruses are significantly closer to morbilliviruses than to any other virus genera (bootstrap values higher than 80%). The same observation was obtained when grouping the viruses through full-genome sequences (Lwamba *et al,* 2005).

In our work, the initial observation in Ethiopia of a difference in clinical expression after infection of different species by PPRV supported the hypothesis of variable strain virulence. To assess this possibility, we have investigated the cell susceptibility of PPRV and tried to identify the molecular basis for different cell host range and replication capacity of this virus comparatively to RPV and their implication for understanding the pathology and pathogenesis of PPRV infection. Sequence analysis was done on two important virus components, the attachment H protein and the virus promoters. We conclude that the virus has modified nucleotide sequences either in the H gene (responsible for attachment with cellular receptors) or its promoters (which are responsible for initiation of transcription and mRNA replication) virulence/attenuated phenotype.

Recommendations

1. **Epidemiology and Control:** PPR is one of the most important diseases of small ruminants in Africa, where poor farmers with subsistence agriculture rely on these animals for their livelihood and food security. It appears that in Africa, goats are severely affected while sheep undergo a mild form of the disease. PPR is one of the most important economical diseases in Ethiopia, since it had been confirmed in goats in 1991. However, its circulation in other animals has never been described. In the present work, we showed that the antibody seroprevalence in camel, cattle, goat and sheep confirmed natural transmission in these animals without clinical disease. The apparent absence of pathogenicity in these animals may have been due to host resistance or loss of virulence of the virus strain in Ethiopia. The epidemiology of the disease is much more complex than previously thought with added differences in pathogenicity and virulence.

   Therefore, in our view PPR warrants due attention for control and future eradication. PPR can be effectively controlled by RPV vaccine, rinderpest eradication programmes have been launched in many countries and, *Office International des Epizooties* (OIE) recommends the cessation of vaccination of all the animals with RPV vaccine so that any residual foci of RPV could be identified. Under these circumstances, small ruminants could only be protected against PPR by using homologous attenuated vaccine. In addition, the successful use of an attenuated PPRV vaccine against RPV has been reported in goats, opening the possibility to use it as a differentiable vaccine for cattle. Indeed, after RP is eradicated in cattle, small ruminants may serve as a reservoir from which RPV could re-emerge. Since there is presently no test for distinguishing between vaccine and wild-type RPV, one cannot determine the origin

(vaccinal or natural infection) of antibodies in goats and sheep. The problem of differentiating between vaccine and wild-type PPRV may also be posed. This may be the stimulating force for developing marked vaccines for both rinderpest and PPR. Future vaccines should be able to incorporate marked vaccines to differentiate natural infection from vaccine induced antibodies.

Therefore, control programmes of PPR should be supported by field data generated by rigorous epidemiological surveillance, risk analysis and geo-referenced mapping systems using GIS.

2. **Diagnostic tools:** Both antigen detection and cELISAs have been effectively used in diagnosis and surveillance of PPR. Virus isolation has been used as a gold standard technique. Initial studies on the relationship among the morbilliviruses were done using classical serological tests (agar gel precipitation, complement fixation, hemagglutination and virus neutralization) and cross protection studies. Cross neutralization has been adopted as a means for differentiating between PPRV and RPV whose host ranges overlap in small ruminants. Serum raised against one virus neutralised the homologous virus at a higher titre than the heterologous one. A practical consequence of serologic cross reactivity between morbilliviruses is that diagnostic tests based on polyclonal antibody are incapable of distinguishing between PPRV and RPV.

   The capability of cells to be infected and support active virus replication has important implications on the pathogenesis of the disease. Morbilliviruses use SLAMs of their respective host species as cellular receptors. However, MV, CDV, and RPV strains could use SLAMs of nonhost species as receptors, albeit at reduced efficiencies. Thus, the finding that these three morbilliviruses use SLAMs as cellular receptors suggests

that the usage of SLAM as a receptor has been maintained from the ancestral virus, accounting for an essential part of the pathogenesis of morbillivirus infections.

Differences in sensitivity of cells to PPRV and RPV were observed using monoclonal antibody based analysis with the Facs scan flow cytometry. Therefore, more works should be conducted on merits of this technique in terms of sensitivity, specificity and cost to use as a diagnostic tool in developing countries compared to classical virus neutralisation test.

3. **Molecular tools**: Molecular basis for attenuation of the vaccine strain was sought by comparative sequence analysis of the genome and antigenome promoters. Thus, promoters of the peste des petits ruminants (PPR) virus, amplified from different pathological samples of sheep and goat origins, were sequenced and compared with corresponding sequences of PPR vaccine strain and other morbilliviruses. The promoters of morbilliviruses are composed of the leader sequence and the non coding regions of the 3' end of the N gene, and the 5' untranslated region of the L gene and the trailer sequences. Both genome and antigenome promoters complement each other on about the first 16 nucleotides for each virus. The promoters are involved in interaction between the genome and the RNA-dependant RNA polymerase for the initiation of transcription, encapsidation of the genome and mRNA synthesis. It is believed that promoters contain the binding site for the viral RNA dependent RNA polymerase. In the cycle of infection, the RNA dependant RNA polymerase of non-segmented negative viruses as morbilliviruses uses the promoters to synthesize the messengers RNA and later full-length genome copies are made via replication intermediate strands of the genome. Therefore, changes in these regions may have a significant effect on the rate of transcription of viral mRNA or full-length genome replication.

Sequence analysis of genome and antigenome promoter regions detected high similarities between field virus and vaccine strain. There had been vaccine strain specific nucleotide mutations. Whether these mutations are associated with virulence or attenuation needs further research. More striking observation from this work is that promoter regions correlated with lineage specific geographic and phylogenetic analysis previously conducted using the F gene. Therefore, relatively short sequences of promoters could be used for virulence and epidemiological studies of PPR phylogeography and molecular phylogenetic classification of viruses within *Paramyxoviridae*.

4. **More research:** Virulence of a virus to a specific host is multifactorial. From virus point of view it is multigenic and is not related to a single event. Nucleotide mutations observed in the promoter regions have not been tested in cellular models and further in animal models. Therefore, further work is needed to establish the role of these mutations in virulence and pathogenicity of the virus. Full length genome sequences are available for MV, RPV, CDV, PPRV and the dolphin morbillivirus (DMV). These data have been used to establish reverse genetics, a technology critical for negative sense RNA virus research. Interaction between viral H protein and cellular receptors needs more work in light of current developments in reverse genetics and molecular modelling. This study will be helpful to better understand the virus-cell interaction and its receptor effects on viral pathogenicity and epidemiology.

Annexe I

Virus neutralisation protocol

The test can be carried in tissue culture roller tubes as follows:

i) inactivated sera are diluted in a two-fold dilution series and mixed with a stock of virus suspension containing approximately 10^3 TCID50/ml;

ii) the virus/serum mixtures are incubated for 1 hour at 37°C;

iii) 0.2 ml of the mixture are inoculated into each of five roller tubes, followed immediately by 1 ml of vero cell suspension in growth medium at a rate of $2x10^5$ cells/ml;

iv) the sloped tubes are incubated for 3 days at 37°C;

v) the medium is replaced with maintenance medium and the plates are incubated for a further 7 days. The virus challenge dose is acceptable if it falls between $10^{1.8}$ and $10^{2.8}$ TCID50/ml. Any detectable antibody at a dilution of 1/8 is considered to be positive.

The serum neutralization has become more efficient and economical with the use of tissue culture microplates and micro-pipetting systems. Thus, using multichannel pipettor, the serum is diluted serially and the constant amount (10^3 TCID50) of virus is added and incubated at 37°C for 1 h. Still using multichannel pipettors, a constant amount of cells ($2x\ 10^4$ cells/ml) is added to the virus serum mixture. The plate is incubated at 37°C in the 5% CO_2 incubator. The plate is read under microscope for cytopathic effect (CPE) of the virus. Judgement of the results is based on positive and negative control wells. No CPE should be detected in the negative control wells. For the positive controls the record should be similar to ten fold dilutions of the virus. The serum titre is the last dilution for which the test serum neutralizes the virus.

Annexe II

PCR Protocol

PCR can be used to amplify the virus RNA in gum and lachrymal swabs transported in phosphate buffer containing penicillin, streptomycin and fungizone, whole blood and postmortem specimens. In brief, PCR can be carried out as follows: RNA is extracted using the rapid methods (Forsyth and Barrett, 1995, Couacy-Hymann *et al*., 2002) and was conducted in nine hundred µl of lysis solution (6 M guanidine isothiocyanate or 6 M of sodium iodide) and 100 µl of sample suspension were mixed in a 0.5ml tube. The tube is spun in a microfuge. The supernatant is discarded and the pellet washed and eluted in DEPC treated water. The RNA was stored in –70°C until used. The primers were derived from the nucleoprotein and phosphoprotein gene (Couacy-Hymann *et al*., 2002). The reverse transcription reaction (RT) was carried out in a 250 µl tube as follows: 7 µl of the extracted RNA (denatured at 65°C for 10 min) were mixed with 1 µl of DTT, 1µl of random primer pdN6 and 5 µl of cDNA synthesis bulk (First strand cDNA synthesis kit) and the mixture was incubated at 37°C for 1 h. Three microliters of the RT product were used as template for the PCR. In a 250 µl thin wall tube, the cDNA was mixed with the following reagents: 5 µl of dNTP mixture (200 mM for each dNTP), 5 µl of 10X Taq buffer, 1µl of forward and reverse primers mixture (100 pmol/µl for each primer), 34 µl of water and 1 µl of Taq polymerase (1.25 U/µl). Once all the reagents were mixed, the tube was placed into the PCR machine. The amplification was carried out according to the following programme: initial heating step 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and the extension at 72°C for 2 min. (Fig. 1-12). Five µl of PCR products were analysed by electrophoresis on a gel (1.5% Nusieve agarose in Tris-borate-EDTA buffer, pH8.3). The gel was stained with ethidium bromide and visualized by UV fluorescence for the presence of DNA bands of the expected size and are photographed.

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Résumé

La peste des petits ruminants (PPR) est une maladie infectieuse, contagieuse des petits ruminants domestiques ou sauvages. Elle se caractérise par une hyperthermie élevée (autour de 41°C), du jetage, des écoulements oculaires, une stomatite nécrosante, de la diarrhée profuse et généralement une forte mortalité. En Afrique, elle peut avoir différentes incidences cliniques sur les moutons ou les chèvres, depuis l'infection subclinique jusqu'à une infection aiguë létale. En Ethiopie, la PPR clinique est rarement décrite et l'étude de la circulation virale était jusqu'à présent peu développée. Dans ce travail, nous montrons la présence d'anticorps contre le virus de la PPR sur un grand nombre de moutons, chèvres, vaches et chameaux éthiopiens et nous confirmons la transmission naturelle du virus PPR chez ces animaux sans manifestation clinique détectable. Cette absence apparente de pathogénicité peut être liée à une résistance génétique particulière des races de petits ruminants présentes en Ethiopie ou à une variation de la virulence des souches de virus PPR. Afin d'étudier ce dernier point, nous avons entrepris des études in vitro sur des souches isolées en Ethiopie et dans différents pays en comparaison avec une souche vaccinale obtenue par atténuation par passages en série sur culture cellulaire et d'autres souches de morbillivirus.

Dans un premier temps, nous avons testé la capacité du virus PPR à infecter différents systèmes cellulaires. Nous établissons que les cellules VERO (fibroblastes de rein de singe) et 293T (cellules épithéliales de rein humain) permettent la réplication du virus PPR comme celle du virus de la peste bovine. En revanche, les cellules B95a (cellules lymphoblastoïdes B de singe) ne multiplient que le virus de la peste bovine. La capacité d'une cellule à supporter la réplication du virus est de nature à influer son pouvoir pathogène et l'épidémiologie de la maladie. La différence de sensibilité des cellules au virus PPR peut être lié à l'affinité de la glycoprotéine d'enveloppe virale H pour son ou ses récepteurs cellulaires utilisés notamment par le virus de la peste bovine. Pour aborder cette question, nous avons entrepris des comparaisons de séquences au niveau de la protéine H du virus PPR, en lien avec ce qui a été déjà décrit sur d'autres morbillivirus.

Pour compléter cette étude sur la virulence, nous avons séquencé les promoteurs de plusieurs souches de virus PPR et conduit une analyse des mutations pouvant jouer un rôle dans l'atténuation. En effet, les promoteurs viraux des morbillivirus déterminent la transcription des ARNm viraux et la réplication du génome viral : la modification de leur séquence peut donc affecter leur efficacité et influer sur la virulence de la souche concernée. Nous observons 7 mutations sur les promoteurs de la souche vaccinale du virus PPR en comparaison avec les autres souches virulentes. Certaines mutations sont retrouvées sur les autres morbillivirus, d'autres sont spécifiques du virus PPR. De cette approche moléculaire, nous déduisons également l'intérêt d'utiliser les séquences des promoteurs du virus, relativement très variables par rapport au reste du génome, pour mener des études de phylogéographie et de comparaison entre paramyxovirus.

Le document de thèse a été organisé en 6 chapitres. Le premier concerne l'histoire naturelle de la PPR avec la description du virus, du génome, de l'épidémiologie, de la transmission, des symptômes, de la pathologie, de l'immunologie, du diagnostic, de la lutte contre la maladie et des aspects économiques en Afrique sub-saharienne. Le deuxième chapitre traite de la biologie comparative du virus PPR avec les autres morbillivirus. Le troisième chapitre concerne les travaux d'épidémiologie de la PPR effectués en Ethiopie. Le quatrième volet de ce travail reprend les études sur la spécificité cellulaire du virus PPR et la comparaison des séquences sur la protéine H. Le cinquième chapitre expose les analyses de séquence des promoteurs génomique et antigénomique du virus PPR. Enfin, la dernière partie comprend une discussion générale et des perspectives.

Mots-clés : Ethiopie, épidémiologie, Morbillivirus, pest bovine, phylogéographie, PPR, promoteurs, virulence