Thèse

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITE

PhD

Présentée et soutenue

Par

Berhe Gebreegziabher

Le 10 Novembre 2006

Development of Dual Vaccines for the Control of Peste des Petits Ruminants and Capripox Infections of Small Ruminants.

Jury :

PROF D.P. PICAVET	**Directeur de Thèse**
DR S. BERTAGNOLI	**Rapporteur**
DR R. DRILLIEN	**Rapporteur**
DR M. BERGOIN	**Rapporteur**
DR E. ALBINA	**Examinateur**
DR A. DIALLO	**Examinateur**

ACKNOWLEDGEMENTS

The French Ministry of Foreign Affaire in general and the CIRAD-EMVT in particular deserve my special attention and heartily appreciation for providing me the post graduate scholarship grant with all the privileges I enjoyed during my stay in France without whom the realization of the present study into practical would have been a dream.

I am particularly indebted to Dr. Adama DIALLO, my advisor, for his unreserved and enthusiastic moral support, diligence, meticulous and incalculable time devotion in guiding me throughout the years.

I would like to express my deep sense of indebtness to Dr. Emmanuel ALBINA my co-advisor, for his excellent leading quality of the programme, rectifying my manuscripts, giving constructive criticism, and constant encouragement throughout my study.

My deep thanks go to Dr. Geneviève LIBEAU, very kind colleague, available at any time to help me. I appreciate her routine follow up of my administrative papers as well as her constant encouragement.

I am also highly thankful to Professor P.D. PICAVET for very kindly accepting to be Director of the Thesis. I'm very grateful as well to Dr. S.BERTAGNOLI, Dr. R. DRILLIEN and Dr. M.BERGOIN for their dedication and commetment to accept to be Rapporters in the Jury.

It is a very good opportunity for me to forward my special thanks and respect to Mlle. Cécile MINET, for her unreserved help provided and for her keen interest to be to my side when ever I am in need of her rich and experienced technical know-how.

I want to express my heartfelt thanks and appreciation to the whole staff of the Animal health department of CIRAD-EMVT (Santé Animale) for their unforgettable hospitality and supports they gave me and my special thanks goes to M. Olivier KWIATEK, M. Christian LEGOFF, Mlle. Denise BASTRON and Mlle. Colette GRILLET for their unreserved help and moral support provided to me.

Last but not least, my wonderful appreciation goes to my beloved wife, W/ro Elsa Girmay and my kids Abenezer and Delina for their moral support, encouragement and patience during my long time absence realizing this work far away from them.

TABLE OF CONTENTS

Title **PAGE**

Résumé

Dans beaucoup de pays d'Afrique, d'Asie, du Moyen et du Proche Orient, les petits ruminants payent un lourd tribut à deux maladies hautement contagieuses, la peste des petits ruminants et la variole des petits ruminants. La Peste des petits ruminants (PPR) connue aussi dans le passé sous le nom de peste caprine est une maladie virale hautement contagieuse affectant les petits ruminants domestiques et sauvages. Elle est due à un virus de la famille des *Paramyxoviridae* appartenant au genre *Morbillivirus*: le virus de la Peste des Petits Ruminants (PPRV). C'est une affection des chèvres et des moutons semblable à la peste bovine. Elle est caractérisée par une stomatite nécrosante, une entérite et une pneumonie grave aboutissant très souvent à la mort. Elle constitue la maladie la plus importante des petits ruminants dans les endroits où elle est endémique, les pays d'Afrique situés entre le Sahara et l'Equateur, le Moyen et Proche Orient et le Sud-Ouest Asiatique. Dans ces mêmes régions, sévit une autre maladie contagieuse virale, la variole ovine et caprine. Les agents pathogènes en cause, le virus de la variole ovine (clavelée) et celui de la variole caprine, entraînent respectivement chez les moutons et les chèvres une maladie aiguë ou sub-aiguë caractérisée par des lésions de variole généralisées sur la peau et les muqueuses, une fièvre persistante, une lymphadénite et souvent une pneumonie virale. On retrouve des lésions noduleuses uniformément répartie sur les différents lobes des poumons.

Contre ces deux maladies des petits ruminants il n'existe pour l'instant aucun traitement médical curatif. Par conséquent, les seuls moyens efficaces pour leurs contrôles sont les mesures de prophylaxies sanitaires et médicales. Les premières, nécessitant des actions d'abattage des animaux, de contrôles draconiens des mouvements d'animaux, des mises en quarantaine, sont des mesures nécessitant d'importants moyens financiers et une très bonne organisation des services vétérinaires, conditions non disponibles dans la plupart des pays concernés par ces deux maladies, des pays en développement. Aussi dans ces conditions le seul moyen effectif applicable pour limiter l'impact négatif de la PPR et de la variole des petits ruminants sur l'économie est la vaccination. Il existe des vaccins monovalents efficaces contre chacune de ces deux

maladies mais ils sont employés de façon sporadique, généralement, à l'occasion de menaces immédiates d'épidémie. La raison probable de cette attitude des services vétérinaires des pays concernés est le coût élevé des campagnes de vaccination systématique des petits ruminants lié surtout à la logistique importante à mettre en œuvre, ce en comparaison du prix unitaire d'un mouton ou d'une chèvre, ce en ne tenant pas compte du rôle social joué par ces animaux.

Pour notre thèse, nous avons mené un travail dont l'objectif était de développer des vaccins bivalents qui pourraient contribuer à une baisse du coût des campagnes de vaccination contre la PPR et la variole des petits ruminants et thermotolérants, caractéristique liée au virus capripox. Pour cela nous avons inséré dans le génome d'une souche vaccinale de virus capripox, la souche KS1, l'ADN complémentaire (ADNc) des gènes des protéines vaccinantes du virus de la PPR, la protéine de fusion (F) et l'hémagglutinine. Les virus capripoxvirus sont très spécifiques de leurs hôtes, les bovins, les chèvres et les moutons. Ils ne sont pas pathogènes pour l'homme et constituent ainsi un vecteur idéal pour le développement de vaccins recombinants destinés à lutter contre les maladies de ruminants. Le présent manuscrit dans lequel nous rapportons les résultats que nous avons obtenus est composé de plusieurs parties : d'abord un résumé de l'état actuel des connaissances sur la PPR et les varioles des ruminants, ensuite la construction du recombinant capripox-FPPR, puis celle du capripox-HPPR, et enfin une comparaison des promoteurs poxvirus pour la production de la protéine recombinante H-PPR par le virus capripox. Le manuscrit se termine par une conclusion sur l'ensemble de nos travaux et d'une réflexion sur les perspectives pour les produits que nous avons développés.

Summary

Two highly contagious diseases, Peste des Petits Ruminants and Sheep and Goat Pox, constitute main constraints to small ruminants production in many countries in Asia, the Near and Middle East and Africa. Peste des Petits Ruminants (PPR), also known in the past as goat plague, is a highly contagious viral disease affecting domestic and wild small ruminants. It is caused by a virus which belongs to the *Morbillivirus* genus of *Paramyxoviridae* family: the Peste des Petits Ruminants Virus (PPRV). It is a rinderpest-like infection of goats and sheep characterized by erosive stomatitis, enteritis, pneumonia and death. Economically, it is the most important small ruminant disease in areas where it is endemic. In the same regions, there is a second contagious viral disease, Sheep and goat pox. The responsible pathogens, the sheeppox virus (SPPV) and goatpox virus (GTPV), cause acute to sub acute disease of infected sheep and goats respectively. The clinical signs of infection may include generalized pox lesions throughout the skin and mucous membranes, persistent fever, lymphadenitis, and often a focal pneumonia and nodules lesions distributed uniformly throughout the lungs.

There is no curative medical treatment against these two viral diseases. Therefore, the only way of tackling them is by means of sanitary and medical prophylaxis. Sanitary prophylaxis to be effective needs the existence of efficient veterinary services, the implementation of animal movement controls with sometimes the stamping out policy. The cost needed for the effective implementation of these means in a short period is too high for most of countries where these diseases are endemic. Therefore the only way for effective control of PPR and sheep and goat pox in those countries is the medical prophylaxis, i.e. the vaccination. Currently, efficient attenuated vaccines exist against each of these diseases. Unfortunately, in most cases they are used only in face of outbreaks to limit their extension. The cost of the logistic needed for systematic vaccination campaigns of small ruminants against either PPR or capripox may be too high for countries if only the individual economic value of goat or sheep, excluding their social value, is considered. A way to cut down this cost is the use of polyvalent vaccine

which would enable, in one shot, the protection of animals against more than one economic important disease.

The objective of our thesis work was to develop dual vaccines that could be used to protect sheep and goat against both PPR and capripox and also thermostable, a characteristic linked to the capripoxvirus, and thereby would contribute to cut down the cost of vaccination campaigns. For that, the complementary DNA, cDNA, corresponding to the gene of PPRV immune protective proteins, the fusion (F) and the haemagglutinin (H) proteins were inserted into the genome of the attenuated strain capripox virus strain KS1. Such a recombinant vaccine may be thermotolerant, a characteristic of poxviruses and this may improve the quality of the vaccine for its use in hot climate conditions. Capripox viruses are highly host-specific microorganisms. They are not pathogenic to human and their host range is limited to cattle and small ruminants and possibly buffaloes. Therefore they constitute an ideal and safe vector for the development of recombinant vaccines for use against ruminant diseases. The present manuscript in which we report on the results we have obtained during our thesis work is composed of the following different parts: a general introduction, a literature review of PPR and capripox viruses, the construction of two recombinant vaccines FPPR/Capripox and HPPR/Capripox, the comparison of efficacy of three poxvirus promoters in HPPRV.

GENERAL INTRODUCTION

In most of developing countries, small ruminant farming allows to cover the basic necessities of the majority of peasants. Sheep and goats are an essential source of subsistence. They determine the survival of the poor people in these countries. Unfortunately, in part of Asia and Africa regions, the production of small ruminants is threatened by a highly contagious and fatal disease: the Peste des Petits Ruminants (PPR). Another economically important disease is affecting the same regions, the Sheep and Goat Pox. Both PPR and Sheep and Goat pox are transboundary diseases; and they make part of the group of economic important animal diseases which outbreaks should be notified to the World Organisation for Animal Health (OIE for Office International des Epizooties). Because of the high importance of sheep and goats for the poor, the control of diseases which have a negative impact on their productions is a major goal for programmes aimed at poverty alleviation. Live attenuated monovalent homologous vaccines are available for the control of PPR and sheep and goat pox. However, the PPR vaccine is thermolabile and its application requires strict cold chain. In contrast, the live vaccine applied against sheep and goat pox is thermotolerant, inherent property of poxviruses. Both vaccines protect after a single injection and the induced immunity covers at least the economic life of the animals, around three years. Since both diseases are found in the same regions, it could be interesting to use a bivalent vaccine that protects against the two infections and this may promote a wider use of vaccination for their control.

To this objective, we have decided to use a live attenuated capripoxvirus as a vector for expressing protective antigens from PPR virus. We expected that the resultant recombinant would be thermotolerant, a characteristic of poxvirus, and effective in the control of both PPR and capripox after a single inoculation to animals. Thereby it is intended to reduce the cost of vaccination campaigns. While developing such a vaccine, we also conducted a study in order to increase its efficacy by the evaluation of three different promoters and the comparison of their capability to induce protective immunity against PPR.

List of Figures

Description **Page**

Chapter 3:

List of Tables

Description **Page**

List of Abbreviations

ATI	A–type inclusion body
BTV	Bluetongue virus
cDNA	Complementary DNA
CDV	Canine distemper virus
cELISA	Competitive enzyme-linked immunosorbent assays
CIRAD	Centre de Coopération Internationale en Recherche Agronomique pour le Développement
CTL	Cytotoxic T lymphocytes
DMV	Dolphin morbillivirus
EMVT	Elevage et Médicine Vétérinaire des Pays Tropicaux
FACS	Fluorescent activated cell sorting
GP	Genome promoter
hPIV	Human parainfluenza virus
HN	Haemagglutinin-neuraminidase
ISRA	Institut Sénégalais de la Recherche Agronomique
IRR	Internal rate of return
KS-1	Kenya sheep-1 isolate
LB	Lysogenic broth
LSDV	Lumpy skin disease virus
MAb	Monoclonal antibody
MV	Measles virus
MVA	Modified vaccinia Ankara
NDV	Newcastle Disease virus
NPV	Net present value
NVI	National Veterinary Institute of Ethiopia
OD	Optical densities
OIE	Office International des Epizooties
OPD	Orthophenylene diamine
ORF	Open Reading Frame

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PDV	Phocine distemper virus
PFU	Plaque Forming Units
PMV	Porpoise morbillivirus
PPRV	Peste des Petits Ruminants Virus
PS	Synthetic Promoter
RdRp	RNA-dependent RNA-polymerase
RER	Rough endoplasmic reticulum
RNP	Ribonucleoprotein
RNA	Ribonucleic acid
RPV	Rinderpest virus
RSV	Respiratory syncytial virus
RV	Rhabdovirus
SGP	Sheep and Goat Pox
SGPV	Sheep and Goat Pox virus
$TCID_{50}$	Tissue culture infective doses 50
UTR	Untranslated region
VNT	Virus neutralisation test
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
WHO	World Health Organization

CHAPTER ONE

Literature review

PESTE DES PETITS RUMINANTS

Definition & Background

Peste des petits ruminants (PPR), also known as goat plague, is a highly contagious viral disease affecting domestic and wild small ruminants, (Furley *et al.,* 1987). It is caused by a virus which belongs to the *Morbillivirus* genus of family *Paramyxoviridae*: the Peste des Petits Ruminants Virus (PPRV). It is a rinderpest-like infection of goats and sheep characterized by erosive stomatitis, enteritis, pneumonia and death. Economically, it is the most important small ruminant disease, particularly in areas where it is endemic: countries located between the Sahara and the Equator in Africa, the Middle East and the Indian sub continent (Lefevre and Diallo, 1990; Taylor *et al.,* 1990; Roeder *et al.,* 1994 and Amjad *et al.,* 1996). It is in the list of animal diseases to be notified to the World Organisation for Animal Health (Office International des Epizooties, OIE) in case of outbreaks.

The disease was first described in 1942, in Cote d'Ivoire, by Gargadennec and Lalanne (1942). The authors reported on the presence of a highly fatal disease resembling to rinderpest but affecting only small ruminants. Cattle in contact with sick animals of this contagious disease were not affected. Latter on, in 1956, Mornet and his collaborators (1956) showed on experimental animals that the causative agents of rinderpest & PPR were closely related. From their experiment, they suggested that the second virus was a variant of the first one which is better adapted to small ruminants. Other subsequent experiments and studies showed that there were actually two different viruses although closely related but evolving independently in nature (Hamdy *et al.,* 1976; Gibbs *et al.,* 1979; Taylor W.P., 1984; Diallo *et al.,* 1987; 1994).

PPR got different designations in the past, which almost all refer to the clinical signs of the disease: erosive stomatitis, goat enteritis, catarrhal fever of goat and Kata (Nigerian local name that means catarrh in English). The term goat plague was also used due to the reason that the disease was producing high level of mortality in goat populations. Finally, the scientific name unanimously adopted is Peste des Petits Ruminants (PPR).

Symptoms

PPR covers three essential forms: a per acute form, an acute form and a mild form.

The per-acute form is seen in young goats. The incubation period is of two days on average. Then appears a strong hyperthermia (41 – 42 °C) quickly followed by an attack of the general state (prostration, pilo-erection, anorexia). The animal shows oral and ocular discharges. In the first days of the disease, constipation can appear. This will be followed very quickly by profuse diarrhoea. In all cases, the disease leads towards death within 5-6 days after the beginning of hyperthermia. The evolution of this per acute form is so fast that it does not allow the appearance of other evocative clinical signs of the disease.

The acute form, most characteristically, resembles to rinderpest. The incubation period is three to four days and the first clinical signs are identical to those of the per-acute form although they are less intense. The disease develops over a longer period. This allows the appearance of other symptoms absent in the preceding form: thus the congestive lesions of the oral mucosal membrane are replaced by ulcers covered by white necrotic tissues (Figure 1). The pulmonary sign is manifested by dry cough, which quickly becomes purulent. The ocular and nasal discharges are serous at the beginning and later on, become mucopurulent. Breathing becomes difficult because of the pulmonary attack (broncho-pneumonia) and the partial nasal obstruction by thick mucosal secretions (Figure 1). These signs are in fact the results of bacterial complications, generally by infection with *Pasteurella multocida*. Because of this form,

PPR was for a very long time confused with pasteurellosis. Other complications of parasitical origin, such as coccidiosis, can render difficult the clinical diagnosis. Pregnant females abort in most cases. The evolution of the disease ends most often by death (40 - 60 %). Those animals surviving the disease remain immunized for the rest of their life.

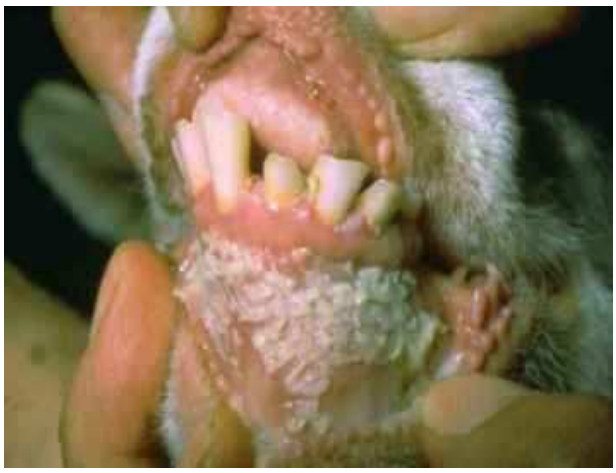
The mild forms are much more frequent than the previous ones and very often they are undetected clinically and are discovery of serosurveillance (Scott, 1981). At the moment of infection animals may have a slight and temporary hyperthermia. Sometimes less abundant ocular and nasal discharges may appear. Dried-up purulent discharges around the nostrils of the animal can be observed and this symptom may lead to confusion with the contagious ecthyma.

Up to 100% of the animals may be affected by PPR in a flock and the mortality rate may range from 0 to 90% according to a) the animal age, young animals over 3 months of age are no longer protected by colostral antibodies anti PPR being highly susceptible to the infection, while old animals which have survived from precedent outbreaks are protected life long, b) the breed, dwarf goats seem to be more affected than sahelian breeds, and c) the species, some findings indicate that sheep are more resistant than goats and others claim the opposite (Shaila *et al.*, 1989; 1996b; Lefèvre P.C. and Diallo A., 1990; Taylor *et al.*, 2002; Diallo A., 2003). For the moment, no study has shown a clear difference in virulence between virus strains so far isolated (Diallo A., 2003).

The carcass of an animal which has died from PPR infection is usually emaciated, the hindquarters soiled with soft/watery faeces and the eyeballs sunken. The eyes and nose contain dried-up discharges and the signs and lesions due to PPRV are listed in the Table 1.

Location	Signs and symptoms
Eye	Profuse catarrhal conjunctivitis, discharges in advanced PPR infection.
Nose	Serous nasal discharge at the beginning, then becoming mucopurulent eroded yellow exudates, partial blockage of the nostrils by dried-up purulent discharges, Small erosions and petechiae
Mouth	Eroded and dead cell on the gums, obscured membrane and nodular lesions.
Lips	Swollen, oedematous, and erosive stomatitis involving the inside of the lower lips.
Respiratory tract	Small erosions and petechiae on the nasal mucosa, turbinates, larynx and trachea
Lungs	Small, dark red/purple areas, firm to the touch, in the anterior and cardiac lobes of the lungs.
Small intestine	Congested, eroded and haemorrhagic.
Large intestine	Congested, in the form of Zebra stripes in the posterior part of the colon.
Lymph nodes	Enlarged and oedematous.

Table 1: Classical signs and lesions of PPRV infection.



A B

Figure 1: Illustration of clinical signs induced by peste des petits ruminants. Classical Mucopurulent nasal and mouth discharge (A); Erosive stomatitis with dead cells on the gums involving the inside of the lower lip (B)

Aetiologic agent and its structure.

The virus which causes PPR belongs to the *Morbillivirus* genus of the *Paramyxoviridae* family. It is closely related to the rinderpest virus, the measles virus of humans, the distemper virus of dogs and some wild carnivores, and the morbilliviruses of aquatic mammals. As all members of the family *Paramyxoviridae,* PPRV is an enveloped pleomorphic particle whose diameter varies between 150 and 700 nm, with a mean of 500 nm. For the rinderpest virus particles, this mean size is around 300 nm (Durojaiye *et al.*, 1985; Bourdin P. and Laurent-Vautier A., 1967). This size variation may be linked to the number of nucleocapsids incorporated into the virus particles. In the case of measles virus, this number might be more than thirty (Rager *et al.*, 2002). The nucleocapsid is formed by the viral genomic RNA, a single-stranded RNA of negative sense, wrapped by the nucleoprotein (N) to which are associated two other viral proteins: the phosphoprotein (P) and the RNA polymerase (L for large protein). It appears as a tube which is long of about 1 µm with a diameter of around 18 nm (Bourdin P. and Laurent-Vautier A., 1967; Gibbs *et al.*, 1979). From the envelope protrude spikes formed by the two viral

glycoproteins which are essential in the first steps of the host cell infection by the virus, the haemagglutinin (H) and the fusion (F) proteins (Diallo, A., 1990).

Viral structural proteins.

- The Nucleocapsid (N) protein

The N protein is the major viral structural protein of morbilliviruses. They migrate on polyacrylamide gels with an apparent molecular weight ranging from 60 for PPRV to 68 kDa for RPV (Diallo *et al.*, 1987). For the latter case, the high apparent MW seems to be linked to the phosphorylation of the protein. No biological significance has been attributed yet to this post-translation modification of RPV N protein. However, for the rabies virus (RV), member of the family *Rhabdoviridae*, both RNA transcription and replication are reduced if the N protein is not phosphorylated (Wu *et al.*, 2002; Yang *et al.*, 1999). Sequence data are now available for all the morbillivirus N proteins. They consist of 525 amino acid residues for RPV, MV and PPRV but only 523 amino acids in the case of CDV, PDV, DMV and PMV (see review in Diallo A., 2003). Sequence comparison of the different N proteins reveals amino acid identities varying from 67 to 74% between the different morbilliviruses. Protein sequence has allowed the identification of four regions with different degrees of conservation: region I (amino acid residues 1 to 120) is quite well conserved with 75–83 % identity across the group, region II (amino acid residues 122–145) shows only a low sequence identity at about 40%, region III (amino acid residues 146–398) is the most conserved with 85 to 90% identity and finally region IV, which is the C-terminal fragment (amino acids 421–523/525), the least conserved part of the protein with about 17 to 30% of homology (Diallo *et al.*, 1994). The nascent N protein when expressed alone, either in mammalian, insect or bacterial cells, quickly associates into nucleocapsid-like aggregates to form a more condensed form of the protein (Mitra-Kaushik *et al.*, 2001). These N-protein aggregates can be detected in both the cytoplasm and the nucleus of the transfected cells. The signal required for nuclear transport (NLS) has been attributed to the leucine/isoleucine-rich motif (TGILISIL) at positions 70-77 of CDV N sequence (Yoshida *et al.* 1999; Sato *et al.*, 2006). At the same position, the N sequence of the attenuated PPRV 75/1 strain is

TGVMISML. The replacement of 3 leucine/isoleucine residues by other amino-acids has probably altered the NLS motif in the PPRV 75/1 N since this protein is never found in the nucleus (A. Diallo, unpublished data).

- *The Phosphoprotein (P) Protein.*

P is one of the three protein components of the Ribonucleoprotein (*RNP)* and acts as a co-factor for the RNA-dependent RNA-polymerase (*RdRp).* It is a multifunctional protein, which binds both the N and L proteins and acts as a chaperone to keep the N in a soluble form for binding to the RNA. The P proteins are much smaller (54–55 kDa) than the values determined from polyacrylamide gels where they migrate at 72–86 Kd (Diallo *et al.*, 1987; Bolt *et al.*, 1995). This aberrant migration can be attributed to the post-translational phosphorylation of the protein which is rich in serine and threonine. PPRV has the longest P protein with 509 amino acids while the DMV P is the shortest with 506 amino acids. MV, RPV, CDV and PDV all have P proteins of 507 amino acids. P is one of the least conserved virus proteins, RPV and PPRV sharing only 51.4% amino acid identity (Mahapatra *et al.*, 2003) with the region extending from position 21 to 306 containing many unconserved residues. Of the three serine residues (positions 38, 49, and 151) identified as potential phosphorylation sites in the RPV P protein, only Ser151 is conserved in all morbilliviruses (Kaushik and Shaila, 2004). The C-terminal region of paramyxovirus P proteins contains the domain that interacts with the exposed C-terminus of the N protein (Ryan and Portner, 1990; Huber *et al.*, 1991). Harty and Palese (1995) have mapped the N binding sites of MV P to two regions: one is present in the N-terminal 100 amino acids, the other being more precisely defined as residues 459–507 at the C-terminus. Using the yeast two-hybrid system it was shown that an N-terminal 60 amino acid region and a C-terminal amino acid region (316–346) are simultaneously involved in an N-P interaction in RPV (Shaji and Shaila, 1999). A more distant C-terminal region of the P protein interacts with L protein in the case of SeV (Smallwood *et al.*, 1994). Furthermore, reflecting the fact that this region has important functions, the C-terminal half of P is more conserved than the N-terminal half, residues 311–418 being the most conserved. The active form of P protein is a tetramer and oligomerization of the protein rather than its phosphorylation, as previously suggested (Kaushik and Shaila, 2004), is

absolutely required for its replication/transcription activity in the RdRp complex *in vitro* (Rahaman *et al.*, 2004). Interestingly, the N protein of most morbilliviruses is found both in the cytoplasm and in the nucleus, but when complexed with the P protein, it is found exclusively in the cytoplasm (Huber *et al.*, 1991; Gombart *et al.*, 1993). The biological significance of this finding remains unknown.

- *The Matrix (M) protein*

The M protein is located inside the viral envelope and is the most conserved protein within the group with identities ranging from 91%, between CDV and PDV, to 76%, between RPV and PDV/CDV, while RPV and PPRV share 84% identity (Table 2). Only a stretch of 20 residues lying between positions 195 and 214 varies significantly between morbilliviruses (Haffar *et al.*, 1999). A pivotal role is played by M protein in the formation and release of paramyxoviruses particles as it serves as a bridge between the external surface viral glycoproteins (H and F) and the nucleocapsid (for review, see Peeples, 1991; Coronel *et al.*, 1999; 2001). It is well established that epithelial cells are polarized, having distinct apical and basolateral domains which are different in structure, for example in lipid and protein composition, and possibly function (Rodriguez and Nelson, 1989). It has been shown with MV that maturation and release of virus particles occur at the apical surface of epithelial cells although the two viral glycoproteins are found mainly at the basolateral surface (Blau and Compans, 1995; Maisner *et al.*, 1998; Naim *et al.*, 2000; Moll *et al.*, 2001; Riedl *et al.*, 2002). The M protein is known to direct to the apical surface of polarized cells the viral glycoproteins which otherwise would accumulate on basolateral surfaces due to a tyrosine-dependent sorting signal located in their cytoplasmic tails. MV viruses bearing a tyrosine point mutation in this sorting signal are unable to propagate by formation of syncytia in polarized cells in vitro and in vivo (Moll *et al.*, 2004). Blood cells such as macrophages which disseminate the virus in the host are likely to be infected upon contact with the basal surface of epithelial cells by direct cell–cell fusion, which is an alternative way for MV to infect cells (Naim *et al.*, 2000; Moll *et al.*, 2001).

- The Fusion (F) protein

The morbilliviruses produce two surface glycoproteins which are embedded in the viral envelope and protrude as spikes: the fusion (F) and the Haemagglutinin (H) proteins. F enables the virus to penetrate the host cell by mediating the fusion of the viral and cellular membranes at neutral pH. For this process to occur, cooperation with the H protein is required since this protein acts in promoting the activity of F (Moll *et al.*, 2002; Plemper *et al.*, 2002). The F gene of all morbilliviruses consists of about 2200 to 2400 nucleotides depending on the virus, 2410 for PPRV F gene. One interesting feature which characterises morbillivirus F mRNAs is the existence of a long 5'-untranslated region (UTR), which is 580 nucleotides long for MV but 634 for PPRV.

The morbillivirus F mRNA translated product, named F0, is composed of 537–552 amino acids residues depending on the virus. Alignment of the F0 from different morbilliviruses reveals high conservation along the whole length of the protein sequence apart from two variable hydrophobic domains (Evans *et al.*, 1994; Meyer and Diallo, 1995). The first is located near the N-terminus and ends at the conserved motif QIHW. This hydrophobic region is predicted to be the signal peptide sequence required for directing the protein through the rough endoplasmic reticulum (RER) and the Golgi apparatus. It is removed by cellular endoproteases after its transfer of the protein to the cell surface. Without the signal sequence all mature morbillivirus F proteins consist of 527 amino acids. The second variable region is located towards the C-terminus and is predicted to anchor the protein into the membrane: the only requirement for this function appears to be that their sequences are hydrophobic in nature. This leaves the extreme C-terminus on the cytoplasmic side of the membrane where it can interact with the M protein. This orientation in the membrane with a C-proximal anchor and a N-proximal ectodomain classifies the F as a type I glycoprotein.

The fusion domain is the third hydrophobic domain in the F protein but, unlike the other two, is highly conserved in all paramyxoviruses. F0 is the inactive form of F. It is activated only upon a proteolytic cleavage to give two sub-units F1 and F2 which remain linked to each other by disulphide bonds. This places the hydrophobic fusion

domain at the N-terminus of the F1 protein. Cleavage of F0 is not required for virus assembly but is critical for the infectivity and pathogenesis in all paramyxoviruses (Watanabe *et al.,* 1995a). For morbilliviruses, cleavage of F0 occurs at the carboxyl side of the pentapeptide Arg-Arg-X1-X2-Arg (X1 being any amino acid but X2 must be either arginine or lysine). This sequence conforms to the minimal consensus sequence Arg-X-X-Arg recognized by furin, an endopeptidase which is present in the *trans*-Golgi network (Watanabe *et al.,* 1995b).

- The Haemagglutinin (H) protein

The H protein enables the virus to bind to the cell receptors, the main being the signalling lymphocyte activation molecule (SLAM also called CD 150), a cell membrane glycoprotein of the immunoglobulin superfamily (Tatsuo *et al.,* 2001). H cooperates with F for the fusion activity of this latter protein (Wild *et al.,* 1991; Das *et al.,* 2000). The H protein varies in length from 604 amino acids for DMV to 617 amino acids for MV while the RPV and PPRV H proteins have 609 amino acid residues. It has only one long hydrophobic domain near the N-terminus (positions 35–58), which acts as a signal peptide but, unlike the signal peptide in the F protein, it is not cleaved from the mature protein as it also functions to anchor the protein in the membrane. The N-terminal 34 amino acids remain on the cytoplasmic side of the membrane while the C-terminus is extruded to the outside. This arrangement, with a N-terminal anchor and a C-terminal external domain, classifies H as a type II glycoprotein. The mature protein is a disulphide-linked homodimer, which probably associates into tetramers in the spike (Plemper *et al.,* 2000; Vongpunsawad *et al.,* 2004). Like the F protein, maturation of H protein occurs by glycosylation, folding and oligomerization as it passes through the RER and the Golgi complex before reaching the cell surface (Hu *et al.,* 1994; Blain *et al.,* 1995; Plemper *et al.,* 2001). The folding is mediated through a transient association of H with cellular chaperone proteins such as calnexin and GRP78 and misfolding of the MV H may result from its retention by the chaperones in the RER and inefficient migration to the cell surface (Bolt, 2001). Reduced transport of the H protein to the cell surface where it can interact with the F protein impairs its capacity to mediate cell-cell fusion (Plemper *et al.,* 2000). Critical cysteine residues at positions 139 and 154 in H-MV were identified

as responsible for its reduced H dimerization and migration to the cell surface. Oligosaccharide side chains are added to the polypeptide in the lumen of the RER, the number of potential N-glycosylation sites on H varying between viruses and within each virus. These side chains are required to move the protein along the exocytic pathway to the cell membrane and the degree of glycosylation may influence the antigenicity of the molecule (Hu *et al.*, 1994). It was shown for the haemagglutinin-neuraminidase (HN) protein of Newcastle Disease virus (NDV) (Panda *et al.*, 2004) that the degree of glycosylation may affect the virus virulence. This has not yet been demonstrated for morbilliviruses.

The H protein, like the P protein, is not well conserved in morbilliviruses. The most distantly related viruses, RPV and PDV, share only 32% amino acid identity and this only rises to about 50% when the two ruminant morbilliviruses, RPV and PPR, are compared (Table 2). This high degree of sequence variation most probably reflects the role of H in binding to host cell receptor(s) and this means that it is also the main target of the host humoral immune responses and virus neutralizing antibody responses are mainly directed against the H protein. For MV, it has been shown that amino acid changes in H that are potentially linked to the host's adaptive evolution are located in B-cell epitopes and at sites linked to its interactions with the cell receptor (Lecouturier *et al.*, 1996; Hsu *et al.*, 1998). B-cell epitopes capable of inducing neutralizing antibodies have been mapped on the H protein to exposed β-sheets on the proposed three-dimensional structure (Langedijk *et al.*, 1997; Sugiyama *et al.*, 2002). Recombination by reverse genetics has shown that, while the H proteins of MV and CDV are interchangeable (Nussbaum *et al.*, 1995; Stern *et al.*, 1995; von Messling *et al.*, 2001), those of RPV and PPRV are not (Das *et al.*, 2000). A number of paramyxovirus glycoproteins have been shown to have both haemagglutinating and neuraminidase activities. However, for the morbilliviruses, strains of MV adapted to grow in tissue culture were the only example within the genus to have haemagglutinating activity, amino acid residues at positions 451 and 481 being critical for the maintenance of this activity (Lecouturier *et al.*, 1996). Some studies have shown that PPRV also has haemagglutination capabilities (Wosu, L., 1985; 1991; Seth and Shaila, 2001). PPRV seems to be the only morbillivirus whose H protein resembles to the haemagglutinin-

neuraminidase (HN) protein of viruses of the Paramyxovirus genera as it has also neuraminidase activity (Langedijk *et al.*, 1997; Seth and Shaila, 2001). The RPV H protein has been shown to have limited neuraminidase activity but it cannot agglutinate red blood cells (Langedijk *et al.*, 1997).

- *The Large (L) protein*

The RdRp (L protein) is the largest virus protein and is also the least abundant. It migrates with an apparent molecular weight of about 200 kDa and is composed of 2183 amino acids for MV, RPV, PPRV and cetacean morbillivirus but 2184 for CDV and PDV (Rima *et al.*, 1986; 2005; Baron MD and Barrett T., 1995; McIlhatton *et al.*, 1997; Martha Y., 2001; Muthuchelvan *et al.*, 2005; Bailey *et al.*, 2005). Considering the size of the L protein, it is surprisingly conserved between morbilliviruses, the percentage identity between the L proteins of RPV and PPRV being about 83% as shown in table 2. CDV and PDV L proteins share the highest percentage of homology with 89.7% of amino acids in common while PPRV L and CDV L share only 57% (Bailey *et al.*, 2005). Comparison of the L protein sequences of morbilliviruses identified three relatively conserved domains separated by less conserved 'hinge regions' (McIlhatton *et al.*, 1997). By the reverse genetics technology, foreign gene like the functional green fluorescent protein (GFP) has been inserted into the L protein at the second hinge region for both MV and RPV but attempts to introduce foreign sequence at the first hinge failed (Duprex *et al.*, 2002). Insertion of the GFP ORF in frame with the L protein sequence produces a green fluorescent virus which grows to an equivalent titre to that of the parent virus in Vero cells; however, this insertion greatly attenuates the RPV pathogenicity for cattle (Brown *et al.*, 2005a).

Because of its size, the RdRp is assumed to carry all the activities necessary for genomic RNA replication and transcription into functional mRNA. All deletion/site mutation studies carried out so far on the protein of different non-segmented negative-sense RNA viruses indicate that the three conserved domains perform functions of the protein (Malur *et al.*, 2002; Cartee *et al.*, 2003). The first domain, residues 1 to 606, has the sequence KEXXRLXXKMXXKM at position 535–549, which is thought to be the RNA binding motif. The second domain, residues 650–1694, contains the sequence GDDD,

flanked by hydrophobic regions, which is thought to be the functional polymerase site of RdRp while the third domain, residues 1717–2183, has kinase activity and may also act as an ATP binding site (Blumberg *et al.*, 1988).

The L protein can only function as an RdRp when it is associated with its cofactor, the P protein. Horikami *et al.* (1994) mapped the binding site for the P protein of MV to the N-terminal 408 amino acids of the L protein, part of domain I. The sequence ILYPEVHLDSPIV, at positions 9 to 21 of morbillivirus L proteins, is partially conserved within the *Paramyxoviridae* family and mutations/deletions involving the sequence ILYPE reduces the binding of P protein to the human parainfluenza virus type 3 (hPIV3) L protein and also reduces its transcription function (Malur *et al.*, 2002).

Virus non-structural proteins

- *The C protein*

In addition to the six structural proteins mentioned here above, paramyxoviruses can also produce a range of non-structural proteins in infected cells. These are encoded by the P transcription unit in alternative reading frames. The first of these, the C protein, is generated by translation of the P mRNA from another open reading frame (ORF) different from the one encoding P protein by use of a second initiation AUG codon which in the case of PPRV, RPV and RPDV, is at 19 nucleotides downstream from the first. This second initiation codon is accessed by skipping the first one due to leaky scanning as neither of these first two AUG codons (CCGAUGG and ACCAUGU, respectively) is in an ideal Kozak context (Kozak, M. 1986). The C protein is a small basic protein with a molecular weight of 19–21 kDa. For both RPV and PPRV it is composed of 177 amino acids, three residues longer than those of CDV and PDV (Barret et al., 1985; Curran, M.D. and B. Rima, 1992; Baron *et al.*, 1993). The MV C protein with 186 amino acids is the longest whilst DMV, with only 160 amino acid residues, is the shortest (Bellini *et al.*, 1985; Bolt *et al.*, 1995). Sequence alignment reveals a high degree of conservation between the different morbillivirus C proteins at the C-terminus (Mahapatra *et al.*, 2003). While the P protein is phosphorylated and found only in the cytoplasm in association with the nucleocapsids, the C is not phosphorylated and can be detected in both the

nuclear and cytoplamic compartments of MV infected cells (Bellini *et al.*, 1985; Alkhatib *et al.*, 1988). However, RPV C protein is uniformly distributed into the cytoplasm and is not found in the nucleus (Sweetman *et al.*, 2001). Interactions of the morbillivirus C proteins with other virus proteins have been studied but the results are not consistent. The RPV C protein self interacts as well as binds to the L protein (Sweetman *et al.*, 2001), indicating a function in modulation of RdRp activity. Other workers could not demonstrate an interaction either with itself or with any of the other viral proteins in MV infections (Liston *et al.*, 1995). As the C protein has an unusual high isoelectric point, creating a strong positive charge at physiological pH, a possible interaction with RNA has been suggested (Radecke and Billeter, 1996). C functions are very poorly understood in terms of their biological significance, although there is evidence that C is a virulence factor for MV (Patterson *et al.*, 2000). Using reverse genetics techniques it has been possible to produce viruses that do not express protein C and MV C-minus mutants show no reduction in viral multiplication nor in the formation of progeny virus in Vero cells. However, they produce reduced number of progeny virus in human peripheral blood mononuclear cells, a normal target cell for MV (Radecke and Billeter, 1996; Escoffier *et al.*, 1999). A RPV C knockout virus, however, showed impaired growth in Vero cells together with reduced viral RNA synthesis (Baron and Barrett, 2000). SeV C-minus mutants show impaired viral replication *in vivo* (Tapparel *et al.*, 1997) and prevention of expression of all the four variants of the C protein in this virus results in severe attenuation of growth in tissue culture and the abrogation of pathogenicity in the natural host (Kurotani *et al.*, 1998). Thus C seems to act as virus virulence factor and in the case of MV it was shown to be an interferon antagonist (Shaffer et al., 2003). Another function of C protein as infectivity factor might be to stabilize virus particles and therefore sustains the viral infection. It is therefore necessary for the efficient virus replication (Devaux P and Cattaneo R., 2004; Takeuchi *et al.*, 2005; Von Messling et al., 2006).

- *The V protein*

This protein is translated from a mRNA produced from the P gene by addition of one or more non-template G residues, depending on the paramyxovirus, during the

transcription process. For all morbilliviruses, there is only one extra G insertion at postion 752 in a highly conserved site (5'UUAAAAAGGGCACAG) named editing site (Baron *et al*., 1993; Blixenkronemoller *et al.*, 1992; Cattaneo *et al.*, 1989; Mahapatra *et al.*, 2003). This non-template G residue addition in the mRNA results in a frame shift of the open reading frame after the extra nucleotide insertion point. As a result, V is shorter than P. Both have an identical N-terminal but different C-terminal protein sequences. For PPRV, V is composed of 298 amino acid residues with a predicted molecular mass of 32.3 kDa. It is one amino acid shorter in length than the corresponding proteins of CDV, PDV, RPV and MV, whereas DMV has the longest V protein (303 amino acids) of all morbilliviruses (Mahapatra *et al.*, 2003). The RPV V protein is phosphorylated and like P, can bind to both N and L, indicating involvement of this protein in the regulation of viral RNA synthesis (Sweetman *et al.*, 2001). V has also been shown to bind unassembled N, but not encapsidated N of other paramyxoviruses (Watanabe *et al.*, 1996; Horikami *et al.*, 1996; Randall and Bermingham, 1996). This means V may bind to N to keep it soluble prior to encapsidation as suggested ealier by Precious *et al*. (1995). Similar studies with MV using the yeast two-hybrid system, failed to show a V–N interaction (Liston *et al.*, 1995). These discrepancies may be due to the insensitivity of the systems used for detecting protein–protein interactions. The C-terminal region of V protein is highly conserved among morbilliviruses and nine amino acids are shared at the editing site, including seven highly conserved cysteine residues. These, along with a number of other residues, are conserved in all paramyxoviruses wherever V protein expression has been shown. This arrangement of cysteines at the C-terminus of V is similar to motifs found in metal ion-binding protein and, in fact, this domain of MV and the Simina virus 5 (SV5) V proteins has been shown experimentally to bind to zinc ions (Liston and Briedis, 1994; Paterson *et al.*, 1995). It has also been reported that the cysteine-rich motif interacts with a host cell-derived protein, the 127 kDa subunit of the damage-specific DNA binding protein (DDB1) (Lin *et al.*, 1998), although the significance of these interactions for the function of the V protein is not clear.

The presumed role of V in regulating RNA synthesis has been studied by abolishing its expression after alteration the editing site of the virus, mutation not affecting the P protein expression. SeV lacking V grows to a comparable titre, and with a similar phenotype, to

wild-type virus in tissue culture cells (Delenda *et al.*, 1997) but displays attenuated replication and pathogenesis in mice (Kato *et al.*, 1997). Mutant of RPV and MV lacking V are also viable in tissue culture (Schneider *et al.*, 1997; Baron and Barrett, 2000). The absence of the V protein enhances viral replication in MV while over-expression attenuates RNA synthesis (Tober *et al.*, 1998) indicating a regulatory role of V protein in the transcription process. However, *in vivo* studies with the MV V-minus mutant showed reduced RNA synthesis and a reduced viral load in a mouse model with a consequent reduction in pathogenicity (Patterson *et al.*, 2000). Baron and Barrett (2000) also reported *in vitro* an enhanced synthesis of RPV genome and antigenome RNAs in a V-minus mutant of the vaccine virus but no studies have yet been carried out to establish the role of V in pathogenesis. It is demonstrated now that V protein is a highly inhibitor of interferon actions and thereby contributes to the immunosuppression induced by morbillivirus infections (Palosaari *et al.*, 2003; Takeuchi et al., 2003; Ohno *et al.*, 2004; von Messling et al., 2006; Nanda SK and Baron MD, 2006).

Virus	**Genome**	**N**	**P**	**C**	**V**	**M**	**F**	**H**	**L**
Peste des petits ruminants	66.98	79.66	58.65	54.02	54.61	89.82	82.74	59.14	83.44
Measles	70.50	82.70	65.35	65.34	64.55	92.26	85.98	67.33	87.73
Canine distemper	63.58	75.38	53.57	52.91	44.67	83.93	78.75	46.26	81.05
Dolphin morbillivirus	65.91	79.69	57.91	55.29	50.50	89.29	84.52	57.14	83.29

Table 2: Percentage similarity of the RPV Kabete 'O' strain to other morbilliviruses

Genome

The genome of morbilliviruses is a single-stranded RNA of negative sense. Its entire sequence has been determined for nearly all of them. PPRV has the longest genome with 15948 nucleotides while that of CDV, composed of 15702 residues, is the shortest (Barrett *et al.,* 2006). For all morbilliviruses, as for many paramyxoviruses, the number of nucleotides composing the entire genome is in agreement with the so-called "rule of six" (Calain and Roux, 1993; see review by Kolakofsky *et al.*, 1998). This rule reflects the fact that each N protein molecule associates with exactly 6 nucleotides of the genomic RNA. This latter should be entirely encapsidated by N for its efficient transcription/replication. The physical map of morbillivirus genome is similar to that of respiroviruses and henipaviruses (see figure 2). It is organised into six contiguous, non-overlapping transcription units corresponding to the gene of the six structural viral proteins in the order of 3'-N-P-M-F-H-L-5' in the genome sense (Rima *et al.*, 1986; Sidhu *et al.,* 1993). They are separated by short sequences of three nucleotides called intergenic regions (IG) which is CTT in most cases. In some virus strains the H-L junction sequence is CGT (Crowley *et al.,* 1988; Baron MD and Barrett T., 1995). Each transcription unit starts by the AGGA sequence (AGGG for the F gene) and ends by a stretch of at least four (A)s that will serves as a signal for the mRNA polyadenylation by the polymerase in a "slippage" manner. At each end of the genome, there is a short sequence of 52 and 37 nucleotides (but 38 for CDV and PDV) respectively at 3' and 5' ends and named leader for 3' end, and trailer for 5' end. The L-trailer junction sequence is CTT for RPV and MV, CTA for PPRV and CAA for DMV, CDV and PDV. The main feature of the genome organisation that is unique to morbilliviruses is the existence of a long untranslated region (UTR) at 3' end of M gene and at the 5' end of F gene (Meyer G. and Diallo A., 1995; Haffar *et al.,* 1999). Both regions, in combination with the IG separating the two genes, make up a continuous untranslated fragment of about 1Kb of nucleotides long with high GC content. It has been shown that these long UTRs are not essential for the virus replication. However, they act as modulators of M and F productions to increase the replication of the virus but minimize its cytopathogenicity at the same time (Evans et al., 1990; Cathomen *et al.,* 1995; Takeda *et al.,* 2005). It is speculated that reduction of

the cytopathogenicity may be advantageous for the fitness and the survival of virus in nature.

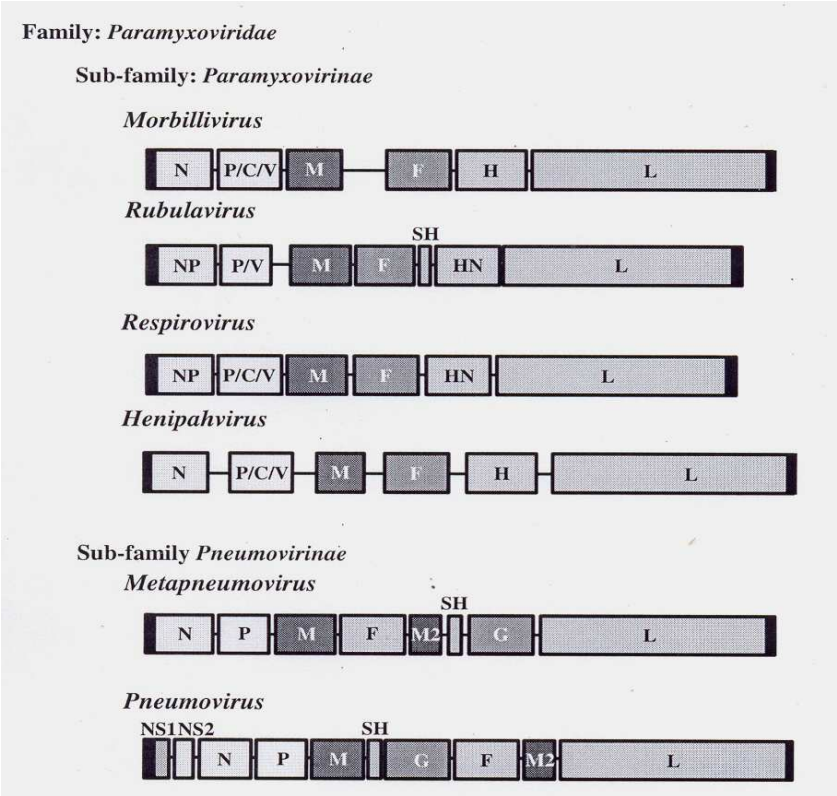


Figure 1

Figure 2: Genome organization of representative members of each genus of the family *Paramyxoviridae*. For some members of the pneumoviruses, the M2 and L ORFs overlap. Gaps between each ORF are also shown although not to scale. Each ORF is denoted by an abbreviation for the protein as detailed in the text. For the rubulaviruses and the pneumoviruses and metapneumoviruses as SH (small hydrophobic) gene is present. The pneumoviruses also have separate NS (non-structural) genes located upstream of the N protein gene. (from Barrett *et al.*, 2006.)

Transcription

Transcription of the genomic RNA by the viral RdRp starts after the release of the nucleocapsid into the cytoplasm following fusion of the virus envelope with the cell membrane. How the RdRp accesses the template RNA is unclear since the association of the nucleocapsid protein with the RNA is intimately strong; it can resist even the high salt conditions required for caesium chloride (CsCl) density gradient purification. Access is thought to be made possible either by a reversible transition in the protein-RNA association or by local displacement of N during transcription, as it occurs in the separation of the two strands of DNA during its transcription (for review, see Kolakofsky *et al.*, 1998).

For negative RNA viruses, to synthesize RNA the RdRp can attach to the genomic RNA only at the level of the genome promoter (GP) which is formed by the leader and the sequence corresponding to the 5' untranslated region of the N protein mRNA (Tapparel *et al.*, 1998; Whelan and Wertz, 1999; Mioulet *et al.*, 2001; Whelan *et al.*, 2004). From there, transcription begins and the RdRp proceeds sequentially along the genomic RNA in a 'stop–start' mode, each transcription unit being defined by the trinucleotide intergenic motif (IG) which is not transcribed normally as it serves as a stop signal. A non recognition of this signal during the transcription step will result in the production of polycistronic mRNAs which contain the IG sequence (for review, see A. Banerjee, 1987; Horikami SM and Moyer SA, 1995). In transcription mode, access of the RdRp to the downstream transcription unit is therefore entirely dependent upon completion and release of a newly synthesized copy of the mRNA from the preceding unit. If the RdRp becomes detached from the RNP template during transcription it must return again to the GP to reinitiate mRNA synthesis. This leads to a transcriptional gradient where less mRNA is transcribed when the gene is more distant from the GP. A progressive decline in synthesis of mRNA occurred with a 20–30% loss of transcription efficacy across each gene junction as it has been shown experimentally with VSV where, using reverse genetics, the positions of the transcription units along the genome were changed relative to the GP (Wertz *et al.*, 1998, Wertz and Ball, 2002). This is probably a

way for the virus of controlling the amount of each protein produced. It results in the higher expression of the N protein, the most abundant protein in virions, and the lower production of the L protein (RdRp) which is only required in catalytic amounts.

All mRNAs are synthesized as naked RNAs and are capped at their 5' ends and polyadenylated at their 3' ends by the virus encoded polymerase so they are stable and efficiently translated by the cell ribosomes. Experiments carried out with VSV showed that the conserved trinucleotide (AGG) at the start of the mRNAs is critical for efficient gene expression and contains essential signals for the correct processing of the nascent mRNA. In its absence, the majority of transcripts are prematurely terminated (Stillman *et al.*, 1998, 1999). In addition, this conserved trinucleotide probably forms part of the signal for capping and methylation of the mRNAs. The conserved tract of four U residues, which always precedes the IG, signals the polyadenylation of the mRNA transcripts. These U-rich sequences are integral to the polyadenylation signal and are the point where 'stuttering' of the RdRp allows hundreds of A residues to be added to the ends of mRNAs. This polyadenylation signal may have other functions, at least in VSV, as it does not only indicate the end of the upstream mRNA, but also appears necessary for efficient transcription of the downstream transcription unit (Hinzman *et al.*, 2002).

Replication

At some point after infection, the viral RdRp starts the genome replication. This occurs probably through a conformation change induced by the attachment of N on the leader sequence of RNA under synthesis, the RNA polymerase doesn't recognize the stop signal of IG. This will result in the synthesis of full copy of genomic to antigenomic RNA or full-length RNA of positive sense (for review, see A. Banerjee, 1987; Horikami SM and Moyer SA, 1995; Whelan *et al.* 2004). The trailer and the 3'untranslated region of the L protein together resemble to the genomic promoter. They form the antigenomic promoter (AGP). The antigenomic RNA, like the negative sense genome RNA, is always encapsidated by the N protein since both the GP and AGP sequences contain RNA encapsidation signals. As a consequence, synthesis of full-length antigenome RNA and N protein production must be linked (Gubbay *et al.*, 2001). Therefore, for the replication process association of three viral proteins namely

P, L an N are absolutely required. Although morbilliviruses are closely related and that both N and L are highly conserved within the group, a study has showed that RNA synthesis is efficient only when the three proteins N/P/L come from the same origin, either RPV, or MV or CDV (Brown *et al.,* 2005b). The precise mechanism triggering the switch from the transcriptive to the replicative mode is not clearly understood. However while GP serves for both the transcription and the replication of the genomic RNA, AGP is not bi-functional since it is exclusively used by the RNA polymerase for the replication of the antigenomic RNA into the genome, the negative sense RNA (Whelan S.P.J and Wertz G., 1999; Tapparel and Roux, 1996; Tapparel *et al.,* 1998; Mioulet *et al.,* 2001; Whelan *et al.,* 2004). The transition from transcription to the replication process by RdRP is still on debate. In the 80's, a self-regulatory model was proposed (Blumberg and Kolakofsky, 1981; for review see Banerjee, 1987). In this model, which was widely accepted for many years, the accumulation of unassembled N proteins would play the major role in switching the RdRp between its transcriptase and replicase functions. This model envisaged a mechanism whereby the RdRp starts transcribing the RNP into mRNA and in presence of sufficient amount of N protein molecules which bound to newly formed RNA, the RdRp switches to the production of a full antigenome RNA. In more details, owing to the absence of sufficient N for binding nascent RNA, RdRp recognizes the transcription control sequences at the leader–N junction, releases the leader and initiates the synthesis of N mRNA. Then, RdRp transcribes the successive genes, recognizing all of the *cis*-acting stop, polyadenylation, IG and start sequences, to produce a full set of viral mRNAs. Once a sufficient concentration of N is achieved, the signal(s) at the leader–N junction would be masked, perhaps by RNA encapsidation with the N protein, thus driving the switch in RdRp activity/function to its replicative mode to begin synthesis of an antigenome RNA. This antigenome is subsequently used as template for the production of new genomes. This theory is in agreement with the observation made by Wertz *et al.* (1998) in which, if the N protein transcription unit is moved further downstream into the genome, less N protein is produced and genome replication is reduced. However, increasing the amount of N protein produced in a minireplicon system for respiratory syncytial virus (RSV) does not enhance the rate of replication relative to transcription

(Fearns *et al.*, 1997). More recent works have led to revision of the model since it is increasingly clear that the switch mechanism is more complicated. It is now thought that transcription and replication require different sets of accessory proteins to interact with the RdRp to form either a transcriptase or a replicase complex. Gupta *et al.* (2003) and Kolakofsky *et al.* (2004) proposed the existence of two different forms of the RdRp, one which is used for transcription and the other for replication. It has long been understood that both L–P and N–P complexes are essential for the replication process and that association of P with N maintains the latter in a soluble form ready to encapsidate newly synthesized viral RNA, but not cellular RNAs, as the N–P complex requires an encapsidation signal to bind the RNA (Horikami *et al.*, 1992; Spehner *et al.*, 1997). In the Gupta model, the transcriptase complex is formed by a RdRp associated with a L-P oligomer and possible host cell factors and differs from the replicase, which is formed from a tripartite association with the additional involvement of a N-P oligomer. In the Kolakofsky model, the transcriptase complex is proposed to consist RdRp with a L-P oligomer and a free P oligomer, which will bind directly to the genomic RNA while in the replicase complex the L-P oligomer is associated with an N-P oligomer. In both models the N protein plays a major role in determining the replicase activity. The M protein may also be involved in the regulation of RdRp activity and the inhibitory effect of M on VSV transcription has been known for many years (see review by Banerjee, 1987). This inhibition has also been shown to occur in MV and in rabies virus (RV) (Suryanarayana *et al.*, 1994; Ghildyal *et al.*, 2002; Finke S., and Conzelman K.K., 2003; Finke *et al.*, 2003; Reuter et. al., 2006). The inhibitory effect of M seems to be independent of its role in virus assembly and budding, the amino acid residue at position 58 in the RV M protein being critical for this function (Finke and Conzelmann, 2003). The exact mechanism underlying M protein inhibition is not yet fully understood but, if the transcriptase complex is functionally different from the replicase complex, it might target the transcriptase directly and leave the replicase unaffected.

Geographic distribution

PPR was first described in Côte d'Ivoire (Gargadennec L. & Lalanne A., 1942) and then afterm it has been recognized in many of the sub-saharian countries that lie between the Atlantic Ocean and the Red Sea (Lefevre and Diallo, 1990). The affected area extends north to Egypt and south to Kenya, in Eastern-Africa, and to Gabon, in Western-Africa. PPR has not been recognized in most of Northern and Southern-Africa. In 1998, serological survey in the United Republic of Tanzania did not detect any antibodies to PPR suggesting that infection has not extended that far to the south. PPR is present in nearly all Middle Eastern countries up to Turkey (Furley *et al.*. 1987; Lefevre *et al.* 1991; Perl *et al.* 1994; Taylor *et al.* 1990, Ozkul *et al.* 2002). It is also widespread in India and southwest Asia (Shaila *et al.* 1989).

PPRV strains classification has been achieved by sequencing the F and N genes (Shaila *et al.* 1996; Kwiatek *et al.*, submitted for publication). Four geographic lineages were thus identified. Figure 3 represents the geographical distribution of the different genotypes of PPRV, according to the results of sequencing of the variable fragment of the N gene (3' extremity of the gene). There are four groups of phylogeny of which, 3 are located in Africa. The fourth group is the only one present into the Indian sub-continent but it also coexists in the Middle East with the East African group III (Figure 3).

It is still not clear whether the apparent geographical spread of the disease in the last 50 years is real or reflects increased awareness, wider availability of diagnostic tools or even a change in the virulence of the virus. It seems most likely that a combination of factors is responsible for the present knowledge of the disease distribution. It is also known that confusion of PPR with pneumonic pasteurellosis and other pneumonic diseases of small ruminants has precluded and delayed its recognition in some countries.

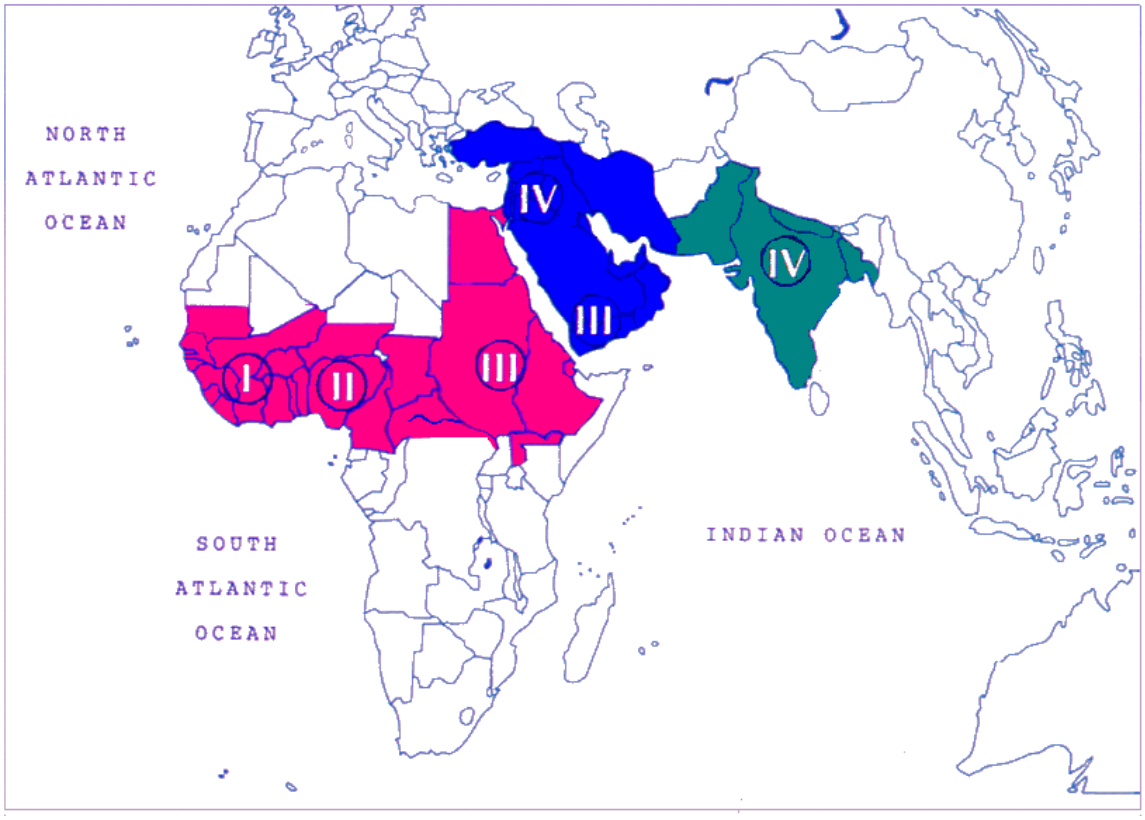


Figure 3: Geographic distribution of Peste des Petits Ruminants. I, II, III & IV correspond to different lineages of PPR distributed around the world.

Economic incidence

Although PPR remains the principal killing diseases of small ruminants in most African, Asian and Middle East countries as recognized in an international survey report published in 2002 (Perry *et al.*, 2002), few economic studies have been made on this disease. The most recent one was published in 1992. It was conducted in Niger (Chip S., 1992). It concluded to an anticipated minimum net present value (NPV) return of 14 millions dollars with an investment of 10 millions dollars and internal rate of return (IRR > 100%) if the cost of the vaccination programme were to be increased five-fold.

Sanitary and Medical Prophylaxis

There is no specific treatment against PPR. Antibiotics may prevent secondary pulmonary infections but this treatment is too costly in case of an outbreak. Therefore the control of this disease is through the implementation of sanitary and medical prophylaxis measures.

Sanitary prophylaxis

Although it is obvious that strict sanitary measures are hardly possible in developing countries where the disease exists, the following measures are recommended to be applied:

- Isolation of infected herds and sick animals for at least 45 days after recovery
- Slaughtering of infected herds (as far as possible)
- Proper disposal of carcasses and products
- Stringent disinfection
- Quarantine before introduction into herds
- Animal and vehicle movement controls within the infected areas

Medical prophylaxis

In the absence of homologous vaccine, and taking advantage of the close antigenic relationship between RPV and PPRV, the attenuated tissue culture Rinderpest vaccine has been used for a long time to protect small ruminants against PPR. At the end of the 80s, a PPRV strain was successfully attenuated by serial passages in Vero cells (Diallo *et al.*, 1989). Demonstrated to be very efficient in the protection of sheep and goats against a virulent challenge, this avirulent PPRV is now widely used in the control of PPR.

The fact that both of the above heterologous and homologous vaccines require effective cold chain to conduct a vaccination campaign, the costs required in poor African countries are enormous. To cut down the cost of vaccination, it would be

advisable to use not only a thermoresistant vaccine but also a polyvalent vaccine for the control of another important disease together with PPR. The thermostability of the current PPR homologous vaccine has been dramatically improved by a new freeze-drying process and addition of stabilizing agents (Worrwall *et al.,* 2001). The objective of our work was to produce such another type of vaccine that would enable protection of sheep and goat against PPR and capripox, another serious disease of sheep and goats in PPR endemic areas. Therefore, to address the above problems, we have used the genetic recombination techniques by taking the cDNA corresponding to the genes of PPR proteins F and H responsible for protective immunity and inserting them in to the genome of capripoxvirus as a vector. Similar work has already been achieved in view of obtaining a recombinant vaccine against Rinderpest disease (Romero *et al.,* 1993; Romero *et al., 1994a*; Romero *et al.,* 1994b). The next part of this document will give some information on poxviruses as etiologic agent of diseases in ruminants and as recombinant vectors for vaccination.

SHEEP AND GOAT POX

Definition

Sheep and goat poxes are acute to subacute diseases of sheep and goats caused by sheep pox and goat pox viruses, respectively. They are characterized by generalized pox lesions throughout the skin and mucous membranes, a persistent fever, lymphadenitis, and often a focal viral pneumonia with lesions distributed uniformly throughout the lungs. Sub-clinical cases may occur. Capripoxvirus infections of sheep and goats result in major economic losses, and place constraints on both international trade and the introduction of exotic breeds to endemic areas (Davies F.G., 1981). They are part of the group of animal diseases to be notified to the World Organisation for Animal Health (OIE: Office International des Epizooties).

Symptoms

Symptoms of sheep and goat pox are identical and therefore are not descripted separately. Usually, three forms of sheep and goat pox are described: per acute, acute and subclinical:

a) The per acute form: is observed mainly on young animals and is characterized by the exacerbation of general signs of any acute infection, the hyperthermia and the depression. Sometimes the animal dies before the appearance of the skin lesions of the disease. In adults, this form is characterized by general eruption of hemorrhagic papules.

b) The acute form: is the most frequent and the most characteristic form of sheep pox and goat pox. It develops in 4 phases: incubation, invasion (the general symptoms phase), eruption then recovery. The incubation phase has a very variable duration, from 4 days to 3 weeks with an average of one week. The invasion or prodromal phase lasts 2 to 4 days and is characterized by general signs of acute infections: fever, depression, inappetence, trembling and accelerated breathing. During this period, different symptoms can appear such as that of rhinitis (sero-mucosal discharge then muco-purulent) and conjunctivitis (photophobia and lacrimation). After this phase, follows the eruption during which the preceding symptoms become blurred. The eruptive phase can take two different forms:

1. Classical pox. It starts with the appearance of circular hyperaemic spots (macula). This lesion progresses to a raised, slightly blanched lesion that presents erythema with edema in the central part of the lesion, the papule. Its preferential location sites are the perineum, inguinal area, scrotum, udder, muzzle, eyelids and axillae. It can also invade the neck, thorax, abdomen and the internal part of hind legs. Then the centre of the lesion becomes depressed and gray (necrotic). It is surrounded by an area of hyperemia. Late in the course of the disease (2 to 4 weeks after the first signs), the lesions become dry, and form scabs. Pox lesions with a transudate, representing the vesicular stage of the lesion, may be noted. These vesicles evolve into pustules. A characteristic feature of pox lesions is that they involve the entire epidermis and dermis and penetrate into the subcutaneous tissue; it looks like a nodule. Depending on the severity of the skin lesion, there may be a scar, an area devoid of wool or hair, after the lesion heals. Secondary bacterial infection may complicate the healing process.

2. Nodular pox or “STONE-pox”. In this form, papules give rise to firm circumscribed nodules with variable diameters that can be as large as 5 cm. They are named “stone pox”. They involve all the layers of the skin and the subcutaneous tissue and upon necrosis and sloughing they leave a hairless scar. They are specially localized on the head, neck, back, genital organs and udder. Sometimes, the nodules are scattered to the whole body. These will persist for several days and even several weeks then will sank themselves and form crusts.

Some other clinical manifestations such as bronchial pneumonia and keratitis can be added to the above-mentioned symptoms following bacterial complication. Abortion due mainly to fever may also occur.

c) Subclinical forms. They generally remain undetected and consist of transient pyrexia and inappetence, with some limited skin lesions.

Some atypical forms of capripox characterized by meningitis signs, agitation and coma, or by gastroenteritis, have been reported.

Aetiological agent

The causal agents of sheep and goat pox are viruses which belong to the Capripoxvirus genus, along with the Lumpy skin Disease virus (LSDV), a pathogen of cattle and possibly buffalo. This genus is part of the subfamily *Chordopoxvirinae* within the family *Poxviridae.* All those three viruses cannot be distinguished serologically and thus they form one serotype. Differentiation can be made between them on the basis of their animal species pathogenicity: LSDV causes disease in cattle, sheep isolates are more pathogenic in sheep than in goat and the opposite is true for goat isolates. However they are some strains which cause serious disease in both sheep and goats (Davies F.G., 1976; 1981; Kitching and Taylor, 1985). The early electron microscopy observations of these viruses suggested that they could be distinguished according to their size (Tantawi and Falluji, 1979; Munz and Owen, 1966). However, Kitching and Smale (1986), analysing intact forms of different capripoxvirus isolates, did not find significant

differences in their external dimensions. Ovoid, all virions have an average seize of 294x273 nm. Not differentiable on the morphology basis or even by serology, capripoxviruses were thus classified according to their animal origin: sheeppox virus (SPV), goatpox virus (GPV) and lumpy skin disease (LSDV) isolated from sheep, goat and cattle respectively. Their genomes are double stranded DNA with terminal repeated sequences at each end (Gershon and Black, 1987). Early molecular studies of these genomes indicated the usefulness of restriction endonuclease analysis for the comparison study of capripoxvirus strains (Black *et al.,* 1986; Kitching *et al.,* 1989). Indeed, although the patterns of capripoxvirus DNA generated by the digestion with Hind III are similar, confirming the close relationship between viruses within the genus, they have some specific differences that can be related to the animal origin with the exception of the Kenya sheep pox isolate KS1 which is in fact a LSD virus (Black *et al.,* 1986). The comparison of these physical maps has also showed that genetic recombination between viruses within a single host might occur in nature (Black *et al.,* 1986; Gershon *et al.,* 1989). Such events might be rare. Indeed, although animal movements and consequently the frequency of contacts between different animals are important in Sub-Saharan Africa, Kitching *et al.* (1989) have shown by Hind III DNA digestion that cattle field isolates of capripoxvirus have been very stable over a period of 30 years. From the genome sequence, capripoxviruses seem to closely resemble leporipoxviruses within the family *Poxviridae* in term of gene content and organisation (Gesrshon and Black, 1989; Tulman *et al.,* 2001). The full genome sequences for some strains of all three capripoxviruses are now available (Tulman *et al.,* 2001; 2002; Kara *et al.,* 2003). They are long of about 151 Kbp and contain 156 putative genes. The sequence data confirm the close relationship between capripox, sheeppox and lumpy skin diseases viruses: they share 96 to 97% nucleotide identity (Tulman *et al.,* 2002). Comparison of these sequences with those of other mammalian poxviruses has shown a high degree of colinearity and amino acid identity in the central region of the genome. This similarity is disrupted in the terminal regions that contain genes potentially involved in viral virulence and host range. Indeed, the main differences between LSDV in one hand and sheeppox and capripoxvirus genomes in the other hand are located in the same areas: nine LSDV genes with likely virulence and host range functions are disrupted in the capripox/sheeppox virus genomes. It is also in the same regions where are identified gene changes between virulent and attenuated strains of the different capripoxviruses (Tulman *et al.,*

2002; Kara *et al.,* 2003). A thorough investigation of genes located in these terminal regions might help developing more effective capripox vaccine.

Geographical distribution

Capripox diseases are endemic in Africa, the Middle East, the Near East and Asia. If lumpy skin disease (LSD) has been reported in Egypt and Israel, it is mainly a disease of African countries south of the Sahara (Ali *et al.,* 1990; Hafez *et al.,* 1992; Greth *et al.,* 1992; Yeruham *et al.,* 1995). Sheeppox and goatpox are more widely spread than lumpy skin disease. Indeed, their endemic areas extend from China to Afghanistan, Turkey, the Middle East, and all African countries north of the Equator. In the 90's, occasional sheeppox outbreaks were reported in Bulgaria and Greece. In Africa, three epidemiological situations of capripox infection are found: while in the region located between South of the Sahara and North of the Equator coexist the three viruses, Southern Africa and North Africa with the exception of Egypt, have only LSD or sheeppox, respectively (Diallo A. and Viljoen G., 2006).

Sanitary and Medical Prophylaxis

Sanitary measures

Capripox diseases are infectious transboundary diseases and the most effective sanitary measure to prevent their introduction into non-infected areas is the control of animal movements. If a new case is confirmed in an area before extensive spread occurs, this area should be quarantined, infected and exposed animals should be slaughtered, and the premises cleaned and disinfected. Vaccination of susceptible animals on premises surrounding the infected flock(s) should be considered.

If the disease has spread over a large area, the most effective means of controlling losses from SGP is vaccination. However, consideration should be given to the elimination of infected and exposed flocks by slaughter, properly disposing of dead animals and contaminated material and cleaning and disinfecting contaminated premises, equipment, and facilities.

Medical measures

In endemic areas, vaccination is an effective means of controlling losses from SGP. Several modified live virus vaccines have been used for protection against SGP (Kitching, 1983; Kitching, 1986a; Kitching, 1986b; Carn, 1993; Bhanuprakash *et al.* 2004). For sheeppox, one of the vaccines which have been widely used is the sheeppox virus Rumania strain. It has been attenuated by serial passages in lamb kidney cell culture (Ramyar and Hessami, 1967; Ramyar 1965 and Sabban, 1957). One of the goatpox live vaccines is the Mysore strain, which was attenuated on goat testis cells.

Davies, (1976) described the isolation of the 0240 capripox virus strain from a sheep. It has been proved that this virus, also named KS1 for Kenya Sheep-1, is in fact a LSDV (Black et al., 1986; Tulman *et al.,* 2002). Attenuation of this isolate, gave rise to a vaccine to control sheep and goatpox (Kitching *et al.,* 1987). The immunity it provides lasts over one year and protects against generalized infection following for at least three years. This vaccine is recommended for the control of capripox in both sheep and goat but not in cattle because it seems to have residual pathogenicity for some breeds of this species (OIE Manual, 2004; Yeruham *et al.,* 1995).
Since 1984, many poxviruses are used as vector to express foreign genes for research purposes or for the development of recombinant vaccines. Because capripox viruses are host specific, the possibility to use their attenuated strains for that purpose was explored successfully in the 1990's.

Poxviruses as expression vectors

Generalities

Poxviruses have been extensively investigated as vaccine vectors because a) they activate both humoral and cellular immunity depending upon the promoter controlling the expression of the immunogen (Coupar *et al.,* 1986; Andrew *et al.,* 1989; Zavala *et al.,* 2001 and Willey *et al.,* 2003), b) have the capacity to accommodate over 25 kb of extra DNA (Smith and Moss, 1983; Merchlinsky and Moss, 1992) and c) they allow simultaneous expression of several foreign genes (Perkus *et al.,* 1985; Carroll *et al.,* 1998; Welter *et al.,* 2000). The possibility to use poxvirus virus as potential vector foreign gene expression was demonstrated for the first time in 1982 simultaneously by Paoletti and Moss groups (Panicali D & Paoletti, E. 1982; Mackett *et al.*

1982). Two years later, this was followed by the development of the vaccinia-rabies recombinant vaccine which express the rabies virus surface glycoprotein (Kieny *et al.,* 1984) and which has been used successfully in rabies control in wild life (Brochier *et al.,* 1995; 1996). Since then numerous strains of vaccinia were engineered to express a variety of antigens from a myriad of bacterial, viral, and parasitic pathogens with subsequent evaluation of the recombinants in both animal models as well as target species. Vaccinia virus (VV) has been particularly used because of its well-defined molecular characteristics and its success in the WHO vaccine programme to eradicate smallpox (WHO, 1980; Moss B., 2001). This success was made possible because, among other features, the vaccine used in this disease eradication programme, the vaccinia virus, a) could be produced efficiently and at low cost in regional centers, b) its ability to retain potency as a freeze-dried preparation, c) its thermotolerance which facilitate a safe storage and transport to remote regions of the globe, d) the ease of its administration (different routes can be used efficiently). However, the use of this vaccine on a large scale led to undesirable effects like necrosis at the injection site, progressive vesicular eruption, encephalitis and sometimes death. To overcome these side-effects, the possibility of using highly attenuated vaccinia as vector for development of recombinant vaccines has been investigated. Such virus strains have been made available and were proved to be very efficient in induction of both humoral and cellular immunity against the recombinant protein. This is the case of modified vaccinia Ankara (MVA) which has lost about 15% of the VV genome containing host range and virulence factors genes. Its replication is defective in human cells (Blanchard *et al.,* 1998).This is the case also for the NYVAC vaccine which derives from the Copenhagen VV strain after deletion of 18 genes from the viral genome and involved in the pathogenicity of the original virus (Tartaglia *et al.,* 1992; Hel *et al.,* 2002).

Still because of vaccinia safety concerns, other investigations were made for use of host specific poxviruses such as avipox viruses. Their productive infection is restricted *in vivo* to avian species and *in vitro* to cells derived from avian species. However, inoculation of mammalian cells with avipox-based recombinants results in expression of foreign genes, and their inoculation to mammals results in the induction of protective immunity (Taylor and Paoletti, 1988; Taylor *et al.,* 1988; Taylor *et al.,* 1992; Cadoz *et al..,* 1992; Kent SJ, *et al.,* 1994; Fries *et al.,* 1996; Stannard *et al.,* 1998; Vazquez-Blomquist *et al.,* 2002; Nacsa J et. *al.,* 2004).

Capripox virus as a vector

Capripoxviruses are also being targeted as vectors for foreign gene expression as they are highly host-range restricted and a number of attenuated strains have been used effectively as vaccines for many years (Kitching *et al.,* 1987; Hunter and Wallace, 2001). The attenuated KS1 strain was used as vector to express either the fusion or haemagglutinin genes of rinderpest virus and the resultant recombinant viruses were used successfully to protect cattle against both rinderpest and LSD (Romero *et al.,* 1993; Romero *et al.,* 1994a; Romero *et al..,* 1994b; Ngichabe *et al.,* 1997). They were used also to cross protect sheep and goats against peste des petits ruminants (Romero *et al.,* 1995). However, the KS-1 recombinant virus expressing the major core structural protein (VP7) of blue tongue virus (BTV) provided only partial protection to sheep against a virulent heterotypic BTV challenge (Wade-Evans *et al.,* 1996). Like KS1, the LSDV vaccine Neethling strain has been used also as a vector to deliver antigens. A LSDV recombinant for rabies glycoprotein has proven to induce immune responses in cattle and in mice (Aspden *et al.,* 2002; Aspden *et al.,* 2003). Identically, a LSDV recombinant for G1 and G2 glycoproteins of Rift Valley Fever induced protection in mice (Wallace *et al.,* 2006).

The capripoxvirus vector has two major advantages over vaccinia when it comes to veterinary vaccine applications. Firstly, it is not a human pathogen and the same safety problems do not arise. Secondly, it is already used as a veterinary vaccine to protect against sheeppox, goatpox and LSD infections (Kitching *et al.,* 1987). Its use as a vector for expressing gene of foreign immune protective proteins offers the possibility to develop multivalent vaccines for ruminants and thereby the possibility to cut down the cost of vaccination campaigns.

Conclusion

Poxviruses as vehicles for gene expression and recombinant vaccines have come a long way in almost 20 years. This period has seen vast improvements in selection strategies, vaccination regimes and applications (Kwak *et al.,* 2004). Now that the genome of many poxviruses has been sequenced, it has become possible to assign functionality to most poxvirus

genes. This has opened up new avenues for the design of improved vectors for use in vaccinology, immunotherapy and cancer therapy (Jackson *et al.*, 2001; Boyle *et al.*, 2004).

In the vaccinology area, and with the view to develop tools that may help in the control of important small ruminant diseases, we have used capripox as host specific vector for expressing PPRV immunoprotective genes and tested the resultant recombinant viruses for their capacity to protect animals against two diseases following a single inoculation.

CHAPTER TWO

Construction of recombinant vaccines

Introduction

As indicated in the previous chapter, PPRV is an enveloped virus which contains two external glycoproteins, the fusion (F) and the attachment or heamagglutinin (H) proteins. These two proteins are needed and are essential in the initial step of the viral infection. This starts by the attachment of the virus to the target cell through the interaction between H and a cell surface protein used by the virus as the receptor. Following this attachment, F mediates the fusion of the viral envelope with the cell membrane to enable delivering the nucleocapsid into the cytoplasm where subsequent steps of the virus multiplication take place (Choppin P.W. and Scheid, A., 1980). By virtue of their functions in the virus replication, the host protective immune responses are directed against (Merz *et al.*, 1980; 1981). It is why we have genes corresponding to these two proteins for the development of recombinant capripox viruses as potential vaccines to protect small ruminants against both capripox and PPR infections.

FPPRV/Capripox recombinant construction

The next article describes the strategy adopted to generate the recombinant capripox/ FPPRV and the results of *in vitro* and *in vivo* expression of the transgene. We have shown that the FPPRV recombinant capripoxvirus, at doses as low as 0.1 PFU, can protect goats against PPR disease despite the fact that an abortive multiplication of the challenge virus may had occurred.

Article 1

JOURNAL OF VIROLOGY, Jan. 2003, p. 1571–1577 Vol. 77, No. 2
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.2.1571–1577.2003

NOTES

Development of a Dual Recombinant Vaccine To Protect Small Ruminants against Peste-des-Petits-Ruminants Virus and Capripoxvirus Infections

G. Berhe,[1] C. Minet,[1] C. Le Goff,[1] T. Barrett,[2] A. Ngangnou,[3] C. Grillet,[1] G. Libeau,[1] M. Fleming,[2] D. N. Black,[2] and A. Diallo[1]*

Cirad, Programme Santé Animale, Campus International de Baillarguet, 34398 Montpellier Cedex 05, France[1]*; Institute of Animal Health, Pirbright Laboratory, Pirbright, Surrey GU 24 ONF, United Kingdom*[2]*; and LANAVET, Garoua, Cameroon*[3]

Received 19 June 2002/Accepted 17 October 2002

A recombinant capripoxvirus vaccine containing a cDNA of the peste-des-petits-ruminants virus (PPRV) fusion protein gene was constructed. A quick and efficient method was used to select a highly purified recombinant virus clone. A trial showed that a dose of this recombinant as low as 0.1 PFU protected goats against challenge with a virulent PPRV strain.

Sheeppox and goatpox are contagious diseases of sheep and goats, respectively, characterized by fever, lacrymation, and secondary bronchopneumonia with nasal discharges. The characteristic lesions are skin macules that evolve into papules, which then progress in some cases to vesicles or nodules. The causal agents are classified in the *Capripoxvirus* genus. Members of this virus group have a host range specific to sheep (sheeppox), goats (goatpox), or cattle (lumpy skin disease), and attenuated vaccines are already available to control capripoxvirus infections (12). Capripoxvirus is therefore an ideal poxvirus vector for the development of recombinant multivalent vaccines to enable delivery of immunogenic genes from other ruminant pathogens that share the same geographical distribution. With that in mind, the attenuated capripoxvirus strain KS-1 was used to develop an effective recombinant rinderpest vaccine expressing the fusion (F) and hemagglutinin (H) proteins of the rinderpest virus (22, 23). This vaccine has now been tested and shown to be effective in long-term trials (19, 20). Capripoxviruses are present in Asia, the Middle East, and Africa. In many of these areas of endemicity, the most important contagious disease of small ruminants is peste des petits ruminants (PPR). It is generally characterized by erosive stomatitis, catarrhal inflammation of the ocular and nasal mucous membranes, diarrhea, and death in 50 to 80% of the acute cases (14). The causal agent, the peste-des-petits-ruminants virus (PPRV), is a member of the *Morbillivirus* genus within the family *Paramyxoviridae* (11). An effective live vaccine is currently in use. It was attenuated by serial passage of the Nigeria 75/1 strain of PPRV in Vero cells (7). As is the case for other morbilliviruses, this vaccine is thermolabile, and it is necessary to maintain it in an effective cold chain, a condition that is sometimes difficult to achieve in many of the developing countries where the disease is endemic. Thus a more heat-stable vaccine would be beneficial for use in countries with hot climates.

PPRV, like other viruses in the family *Paramyxoviridae*, is an enveloped RNA virus with two external glycoproteins, F and H, associated with the envelope. The F protein enables the virus to penetrate the cell membrane and enter the cytoplasm by effecting fusion of the virus and host cell membranes. This phenomenon is also responsible for virus spread from cell to cell without the formation of free viral particles, and this protein is critical for the induction of an effective protective immune response (4, 13, 16, 17). The F proteins of several morbilliviruses have been expressed in different poxviruses by using recombinant DNA technology, and the resultant viruses have proven to be effective vaccines (1, 2, 8, 21, 22, 25, 26, 28–30). Here we report the insertion of the F gene of PPRV into the genome of the attenuated capripoxvirus strain KS-1.

The cDNA corresponding to the PPRV F gene was released by enzymatic digestion from plasmid FNYZ12 as previously described (18). It was subcloned into plasmid JC-35, which contains the vaccinia virus early/late p7.5 promoter. The insertion was made so that, in the new plasmid, FPJ2, expression of the PPRV F protein gene was driven by this promoter. The *Escherichia coli* xanthine-guanine -phosphoribosyltransferase gene (*gpt*) was used as a dominant-selectable marker to isolate the recombinant (3, 9). This gene is under the control of a p7.5 promoter in the plasmid GPT. It was released from that plasmid and inserted into FPJ2 in a way to place the two promoters back to back. From this final construct, the *E. coli gpt*-p7.5-p7.5-PPR F cassette was released by enzymatic digestion and inserted into the plasmid pKH66T at the *Kpn*I site of the gene coding for capripoxvirus thymidine kinase (TK). Indeed, pKH66T contains the *Hin*dIII S fragment of KS-1 DNA, which encompasses the complete TK gene of capripoxvirus KS-1 (10). The final construct was used to generate recombinant

* Corresponding author. Present address: Animal Production Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, IAEA Laboratories, A-2444 Seibersdorf, Austria. Phone: (43-1) 2600 28355. Fax: (43-1) 2600 28222. E-mail: a.diallo@iaea.org.

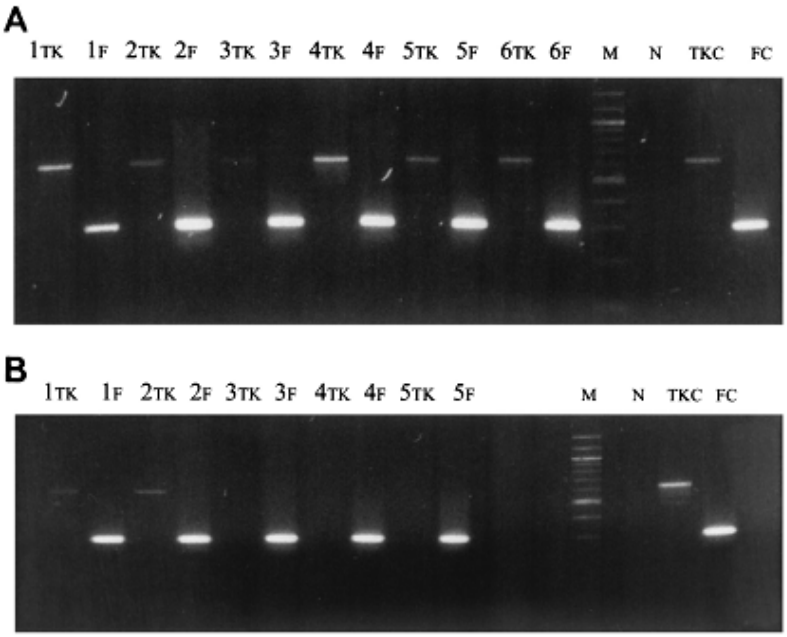


FIG. 1. Screening of the recombinant virus clones by PCR. Primers CPTK7 (5′ ACTTATCAGATTTTGTTACGACATT) and CPTK8 (5′ CGATGAGTTCTATTTCCTTTTCTTTAG) were located in the TK gene of capripoxvirus strain KS-1 at each side of the insertion site *Kpn*I. Primers F1ab (5′ ATGCTCTGTCAGTGATAACC) and F2ab (5′ TTATGGACAG AAGGGACAAG) were located in the PPRV F gene. The PCR products obtained with these primers from samples collected during the different steps of the recombinant virus selection process were analyzed by agarose gel electrophoresis (see the text). The samples amplified with the capripoxvirus TK primers are indicated by TK, while those amplified with the PPRV fusion protein gene primers are in lanes F. M, molecular size markers (100-bp ladders); TKc, positive control capripoxvirus DNA; Fc, positive control PPRV F cDNA; N, negative control (no DNA template). (A) Samples from the first method. Sample 1 is the starting virus stock suspension to be screened for the recombinants. Samples 2, 3, and 4 are from three successive plaque purifications. Samples 5 and 6 are from two successive growth rounds of sample 4 in selection medium. (B) Samples from the second method. Sample 1 is the starting virus stock suspension to be screened for the recombinant. Samples 2 and 3 are from two successive limiting dilution purifications. Sample 4 is a clone collected by plaque purification of sample 4. Sample 5 is aliquot of the virus suspension obtained by one growth round of sample 4 in the selection medium.

viruses with the capripoxvirus KS-1 in lamb testis (LT) cells as described previously (22). The virus suspension obtained following transfection of capripoxvirus-infected cells with the insertion plasmid was used to select the recombinants. This was done in the presence of the *E. coli gpt* selection medium containing mycophenolic acid (MPA) (22). However, while the previous authors were confirming the nature of their clones by DNA probes, we adopted a faster PCR-based method that could be performed directly on the samples without the need for DNA extraction. For that, we used both capripoxvirus TK and PPRV F gene-specific primers (CPTK7 and CPTK8 and F1ab and F2ab, respectively) (Fig. 1). Five microliters of each sample to be tested was used directly in the PCR, which consisted of 10 μl of 10× *Pfu* buffer, deoxynucleoside triphosphates (dNTPs [250 μM each]), 25 pmol of each primer, and 2.5 U of *Pfu* polymerase (Stratagene) in a total volume of 100 μl. The PCR was carried out under the following conditions: a first step at 94°C for 5 min; 30 cycles of amplification, each consisting of 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min; and a final step at 4°C to keep the samples. Fifteen microliters of the amplified products was analyzed by electrophoresis on agarose gels. The primers CPTK7 and CPTK8, located in the TK gene of the capripoxvirus strain KS-1 at each side of the insertion site, amplify a fragment about 600 nucleotides long in the absence of a foreign gene insert. The PPRV F gene insert is about 2,400 nucleotides long and is very GC rich (18); therefore, the expected size of the target to be amplified by the primers CPTK7 and CPTK8 in the recombinant would be about 3,000 nucleotides. The conditions used for the PCR allow amplification of the 600-nucleotide target, but not a target of 3,000 nucleotides. Thus, in the case of a recombinant, the PCR with primers CPTK7 and CPTK8 will be negative, while it should give a product of 300 nucleotides amplified on the PPRV F gene with primers F1ab and F2ab. Figure 1A shows the DNA amplified from samples collected at different steps in the procedures of recombinant selection. As can been seen (lanes 1 to 6), a clone that was obtained was still contaminated with parental virus even after three plaque purifications and two rounds of growth in selection medium. The PCR test was positive with both pairs of primers (lanes TK and F). Similar results were obtained with five other selected clones. We presumed that capripoxviruses have a high ten-

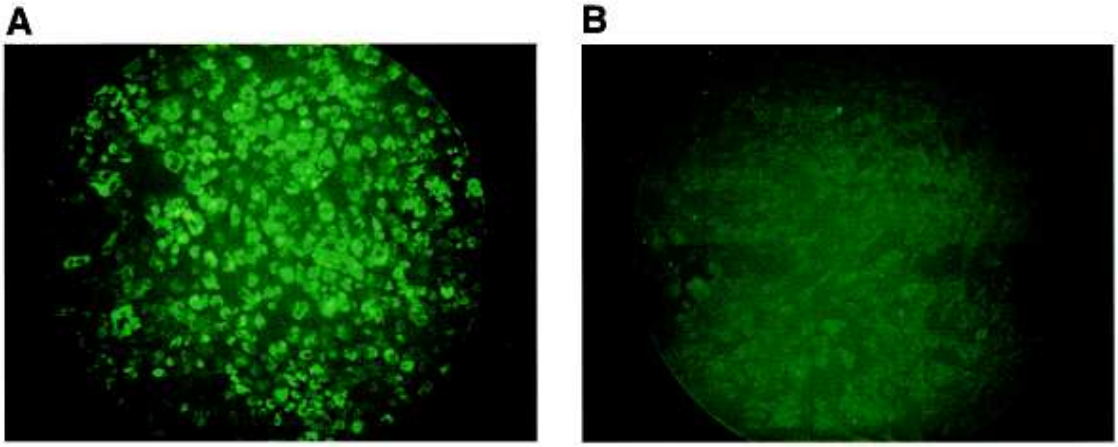


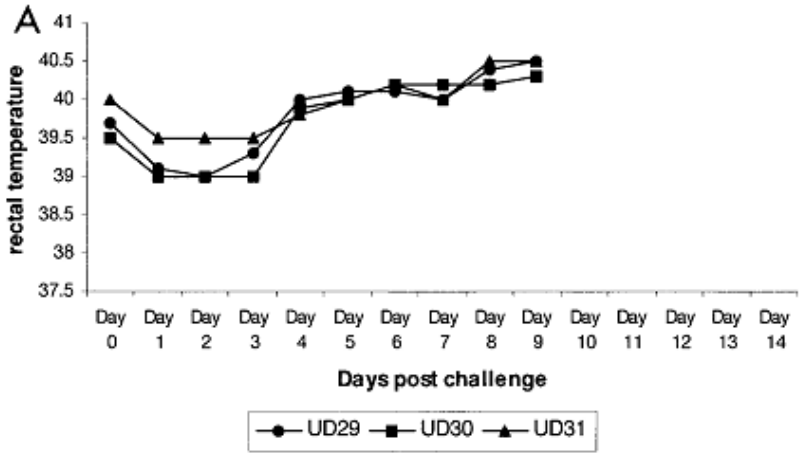
FIG. 2. Detection of the PPRV F protein by immunofluorescence staining. LT cells were infected with the recombinant reCapPPR/F (A) or the parental capripoxvirus KS1 (B). Two days after infection, the cells were submitted to indirect immunofluorescence staining with the anti-PPRV F MAb as described by Romero et al. (22), but without Triton treatment.

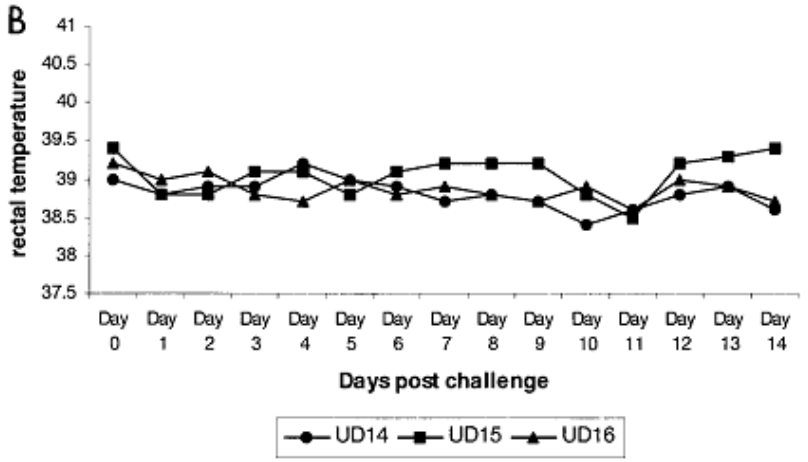
dency to form clumps, and by this means, the recombinant viruses, which can grow in the selection medium MPA, could act as a helper virus and allow replication of parental virus in the same clump. We therefore modified the selection method to include a sonication step. One milliliter of the stock virus suspension was submitted to sonication for 30 s in a water bath sonicator with the power set at 15 W (Ultrasonic Processor, Bioblock Scientific). After sonication, 10-fold serial dilutions of the sample were made from 10^{-1} to 10^{-7}. Confluent LT cells in 96-well plates were preincubated in selection medium for 16 h and then infected with 200 μl of diluted virus suspension (2 wells per dilution). After 10 days of incubation at 37°C, the supernatants from wells from the most dilute suspensions showing a virus cytopathic effect were harvested. These were then subjected to another round of limiting dilution selection and plaque purification. The virus material collected at each step of the purification process was always sonicated to ensure that the virus was not clumped, and its purity was checked by PCR. No DNA amplification was obtained with the TK primers after the second limiting dilution selection (Fig. 1B, lanes 3Tk, 4Tk, and 5Tk), while the presence of the PPRV F protein gene was still detected by primers F1ab and F2ab (Fig. 1B, lanes 3F, 4F, and 5F).

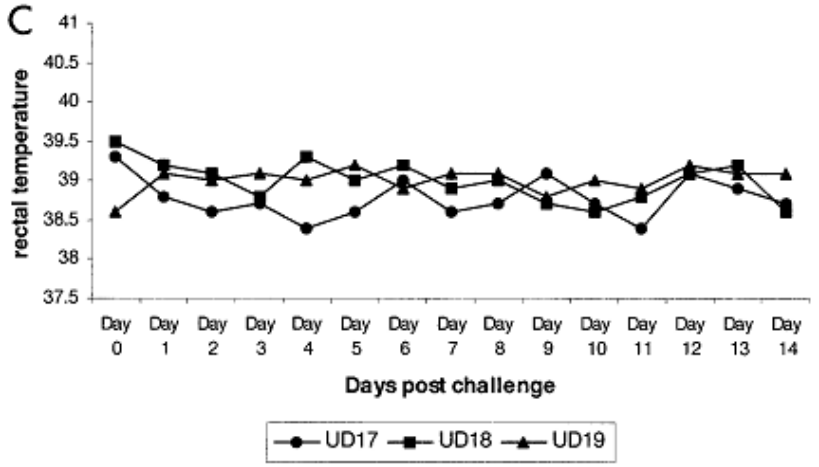
The expression of the PPRV F protein by the pure recombinant virus clone (Fig. 1B, lane 5), was analyzed by immunofluorescence in paraformaldehyde (PFA)-fixed infected LT cells (22), but without treating the cells with Triton. Cells expressing the PPR F protein were detected with a PPRV F-specific monoclonal antibody (MAb) (15) and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin G (IgG). As can be seen in Fig. 2A, cell surface fluorescence was detected on LT cells infected with recCapPPR/F, but not on those infected with the parental capripoxvirus (Fig. 2B).

The recombinant clone recCapPPR/F was tested as a dual vaccine to see if it could protect goats against both PPR and capripoxvirus challenge. For that test, 18 British goats were purchased and housed in six groups of three (groups I to VI). Animals in groups I, II, III, and IV were inoculated with 10^4, 10^2, 10, and 0.1 PFU of recCapPPR/F, respectively. Animals in group V were vaccinated with 10^5 PFU of KS-1 vaccine, while those in group VI were used as unvaccinated controls. One month after vaccination, the animals, with the exception of those of group V, were challenged with virulent PPRV (Guinée Bissau/89 isolate) at 10^4 50% tissue culture infective doses ($TCID_{50}$) per animal by subcutaneous injection. The animals were examined daily for clinical signs of infection, and their rectal temperatures were recorded. All unvaccinated animals developed high fever 4 days after challenge (Fig. 3A). They also developed necrotic mouth lesions, nasal and ocular discharges, and profuse diarrhea. They were euthanized on day 9 because of the severity of the disease. Among the animals that had received the recombinant vaccine, only two developed a transient fever (Fig. 3D). They were in the group vaccinated with a dose of 100 PFU. No other clinical signs were apparent.

Morbilliviruses are lymphotropic viruses and virulent strains induce a severe leukopenia in infected hosts. In the present experiment, leukocytes (WBC) were collected from animals on days 2, 5, 7, 9, 12, and 14 days following challenge and counted. A moderate and transient reduction in the WBC number was observed for a couple of days following challenge in the case of the vaccinated animals (Table 1); however, the unvaccinated animals showed a dramatic leukopenia (reduction of ≥50%). Total RNA extracted from these cells was assayed with a reverse transcription-PCR to detect nucleocapsid protein gene-specific RNA of the challenge virus (5). The quality of the RNA was determined by amplifying a fragment of the cellular β-actin gene by using actin-specific primers. As can be seen in Table 2, PPRV nucleic acid was detected in the WBC from all three unvaccinated animals from day 5 or 7 after challenge. However, in the case of 6 of the 12 vaccinated goats, no DNA amplification could be obtained with the PPRV primers, while


A
rectal temperature
41
40.5
40
39.5
39
38.5
38
37.5
Day 0 Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7 Day 8 Day 9 Day 10 Day 11 Day 12 Day 13 Day 14
Days post challenge
UD29 UD30 UD31


B
rectal temperature
41
40.5
40
39.5
39
38.5
38
37.5
Day 0 Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7 Day 8 Day 9 Day 10 Day 11 Day 12 Day 13 Day 14
Days post challenge
UD14 UD15 UD16


C
rectal temperature
41
40.5
40
39.5
39
38.5
38
37.5
Day 0 Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7 Day 8 Day 9 Day 10 Day 11 Day 12 Day 13 Day 14
Days post challenge
UD17 UD18 UD19

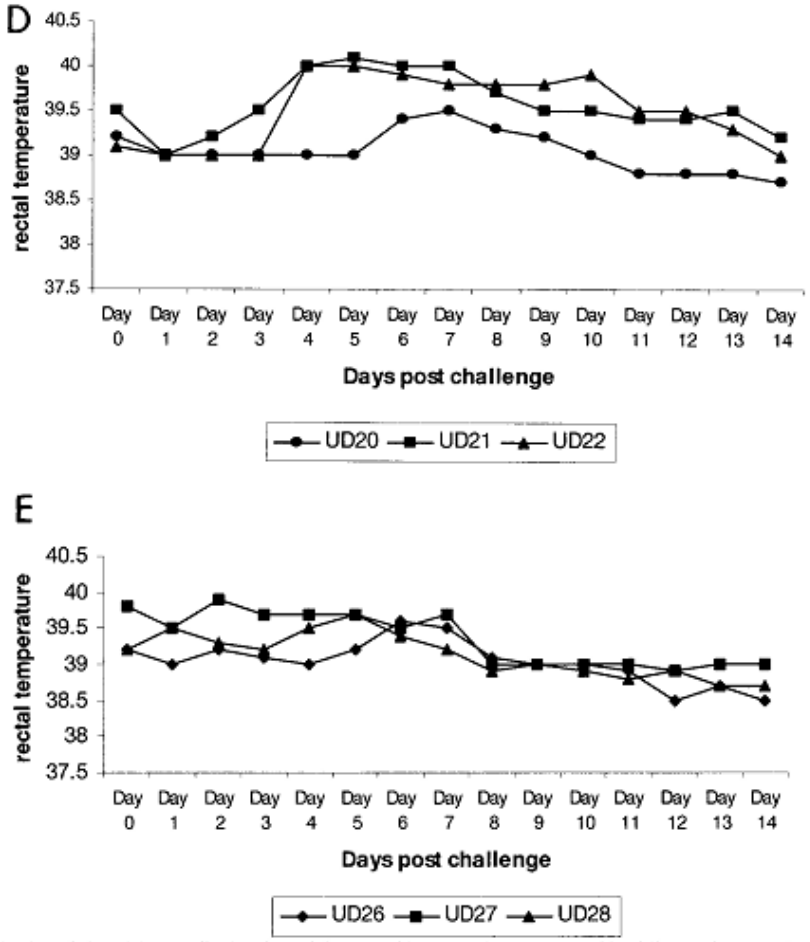


FIG. 3. Determination of the minimum effective dose of the recombinant vaccine represented by daily rectal temperatures of goats following challenge with virulent PPRV. Goats were vaccinated with different doses of the recombinant virus reCapPPR/F and challenged 1 month later with the virulent PPRV Guinée Bissau/89 isolate as indicated in the text. Shown are results for unvaccinated animals (A) and animals vaccinated with 0.1 (B), 10 (C), 100 (D), and 10,000 (E) PFU of the recombinant virus reCapPPR/F.

the same samples were positive for the β-actin gene RNA, indicating the integrity of the RNA extracts. For the remaining six vaccinated goats, PPRV nucleic acid was detected in only one or two postchallenge samples. Blood for serum antibody analysis was collected weekly following vaccination and challenge. The sera were analyzed for PPRV-specific antibodies by using an F protein-based competitive enzyme-linked immunosorbent assay (cELISA) (Table 3). All were negative before challenge. However, following challenge, an anamnestic response was observed after 1 week, indicating that replication of the challenge virus had occurred. One month after PPRV challenge, the surviving animals and those in group V, in addition to three new control goats, were submitted to capripoxvirus challenge by intradermal inoculation of 0.2 ml of virulent capripoxvirus (Yemen isolate). The animals were examined daily for 2 weeks to observe their clinical reactions. The control goats, as expected, developed capripox disease, while all of the others resisted.

The F protein of rinderpest virus has been expressed in both vaccinia virus and capripoxvirus vector systems (1, 22, 30). Here we report the construction of a recombinant capripoxvirus expressing the PPRV F protein. The modifications to the previous selection methodology (22), a sonication step carried out to disrupt potential clumps of virus before the limit dilution followed by PCR screening, allowed a recombinant clone free of parental virus to be selected easily. This recombinant was able to protect goats against both PPR and capripox at a dose as low as 0.1 PFU. With the original capripoxvirus-rinderpest virus F recombinant, a dose of 1.5×10^3 PFU was unable to protect cattle against virulent rinderpest virus challenge (24); apparently it was 10^4 times less effective than the recombinant (reCaPPR/F) reported here. This difference between

TABLE 1. Percentage reduction in WBC counts in goats following challenge with virulent PPRV

Goat	Vaccine dose (PFU/goat)	% Decrease in cell count on postchallenge day[a]:			
		5	7	9	14
UD 29	0	30.6	47.5	48	Dead
UD 30	0	22.8	30.4	**55.6**	Dead
UD 31	0	43.4	49.1	**71.8**	Dead
UD 14	0.1	39	15.4	0	44.5
UD 15	0.1	21	25	15	6
UD 16	0.1	55	39	33	18
UD 17	10	0	0	0	7.8
UD 18	10	0	6.5	23	5
UD 19	10	19.2	31	32	2
UD 20	100	0	6.5	23	5
UD 21	100	13	31.9	10	0
UD 22	100	32.8	44.5	46	0
UD 26	10,000	28.7	0	0	0
UD 27	10,000	37.9	37.3	0	37
UD 28	10,000	0	0	0	13.3

[a] The values are expressed as percent decrease in the cell count from that found on day 2 postchallenge. A decrease of a 50% (boldface) is considered an indication of severe leukopenia.

the two recombinants may be due to the type of promoter used: vaccinia virus p11 in the case of the rinderpest virus F protein and vaccinia virus p7.5 in the case of the PPRV F protein. Tsukiyama et al. (27) developed two rinderpest vaccinia virus (RRV) recombinant vaccines in which the H protein gene was expressed under the control of either the vaccinia virus early/late promoter p7.5 or the cowpox virus A-type inclusion body (ATI) promoter. They reported the induction of a higher immune response with p7.5/RVV than with ATI/RVV in vaccinated rabbits. Both ATI and p11 are strong late promoters. Proteins expressed under the control of such promoters may induce less cellular immunity than those that start synthesis early in the viral replication cycle (6). Another possibility may explain the lower dose requirement of reCaPPR/F than that reported by Romero et al. (24) for rinderpest: that is, the degrees of purity of the recombinant clones may have differed and the original capripoxvirus-rinderpest virus recombinant clone may have been contaminated by the parental virus, since its purity was not checked by PCR, a very sensitive technique able to detect extremely small amounts of parental virus contaminant.

TABLE 2. Detection of PPRV N gene-specific RNA by RT-PCR in WBC collected from goats after challenge with virulent PPRV[a]

Goat	Vaccine dose (PFU/goat)	Result on day postchallenge[b]:									
		2		5		7		9		14	
		Actin	N gene	Actin	N gene	Actin	N gene	Actin	N gene	Actin	N gene
UD 29	0	+	−	+	+	+	+	+	+	Dead	
UD 30	0	+	−	+	+	+	+	+	+	Dead	
UD 31	0	+	−	+	−	+	+	+	+	Dead	
UD 14	0.1	+	−	+	−	+	−	+	−	+	−
UD 15	0.1	+	−	+	−	+	−	+	−	+	−
UD 16	0.1	+	−	+	−	+	−	+	−	+	−
UD 17	10	+	−	+	−	+	−	+	−	+	−
UD 18	10	+	−	+	−	+	−	+	−	+	−
UD 19	10	+	−	+	−	+	+	+	−	+	−
UD 20	100	+	−	+	−	+	+	+	+	+	−
UD 21	100	+	−	+	+	+	+	+	−	+	−
UD 22	100	+	−	+	−	+	+	+	−	+	−
UD 26	10,000	+	−	+	−	+	+	+	−	+	−
UD 27	10,000	+	−	+	−	+	−	+	−	+	−
UD 28	10,000	+	−	+	+	+	−	+	−	+	−

[a] RNA was detected as described by Couacy et al. (5).

[b] A β-actin-specific primer set, bAct1 (5′ ACCAACTGGGACGACATGGAGA) and bAct2 (5′ AGCCATCTCC TGCTCGAAGTC), was used to amplify the β-actin mRNA as a control for the quality of the extracted RNA.

TABLE 3. Serological responses in goats following challenge with virulent PPRV[a]

Goat	Vaccine dose (PFU/goat)	% Inhibition (status) on day:		
		0 postinfection	7 postinfection	14 postinfection
UD 29	0	19 (−)	16 (−)	Dead
UD 30	0	21 (−)	15 (−)	Dead
UD 31	0	16 (−)	17 (−)	Dead
UD 14	0.1	5 (−)	31 (−)	74 (+)
UD 15	0.1	−5 (−)	17 (−)	37 (−)
UD 16	0.1	8 (−)	20 (−)	**71** (+)
UD 17	10	5 (−)	44 (−)	**52** (+)
UD 18	10	−14 (−)	31 (−)	**69** (+)
UD 19	10	4 (−)	46 (−)	**56** (+)
UD 20	100	−9 (−)	39 (−)	**76** (+)
UD 21	100	−10 (−)	15 (−)	47 (−)
UD 22	100	−6 (−)	37 (−)	**74** (+)
UD 26	10,000	22 (−)	**51** (+)	**71** (+)
UD 27	10,000	14 (−)	**56** (+)	**75** (+)
UD 28	10,000	18 (−)	**53** (+)	**65** (+)

[a] Serological responses were measured by cELISA based on the use of an anti-PPRV F protein MAb with the attenuated PPRV as an antigen for coating the ELISA plates. Both the test serum sample and the MAb were used at the final concentration of 1/20 in phosphate-buffered saline diluted 1/5 and containing 0.05% Tween and 0.5% lamb serum (total volume, 100 μl per well). Four wells were used as controls and contained only the MAb in the dilution buffer. For the interpretation of the results, inhibition of 50% or greater was considered a positive sample (shown in boldface).

Capripoxvirus is a highly host-specific virus, the host range being limited to cattle and small ruminants. It is nonpathogenic to humans, and so it is an ideal vector for the development of recombinant vaccines for use against ruminant diseases. The results reported here indicate that a capripoxvirus recombinant that expresses the PPR F protein can protect goats against two diseases that are of great economic importance in many developing countries: PPR and capripox. The possibility of dual vaccines made by such recombinants and providing efficacy at doses as low as 0.1 PFU would dramatically cut the cost of controlling these diseases by vaccination. However, more work is needed to establish the duration of immunity provided by this vaccine and to test its efficacy in presence of antibodies against capripoxvirus or PPRV. This work is now in progress.

This work was supported by a grant from the Ministère des Affaires Etrangères (projet Ravira no. 950222000230750l). G.B. was supported by a fellowship from the Ministère des Affaires Etrangères.

We thank Gerrit Viljoen (OVI, South Africa) for helpful discussions during the course of this work.

REFERENCES

1. **Barrett, T., G. J. Belsham, S. M. Subbarao, and S. A. Evans.** 1989. Immunization with a vaccinia recombinant expressing the F protein protects rabbits from challenge with a lethal dose of rinderpest virus. Virology **170**:11–18.
2. **Belsham, G. J., E. C. Anderson, P. K. Murray, J. Anderson, and T. Barrett.** 1989. Immune response and protection of cattle and pigs generated by a vaccinia virus recombinant expressing the F protein of rinderpest virus. Vet. Rec. **124**:655–658.

3. **Boyle, D. B., and B. E. H. Coupar.** 1988. A dominant selectable marker for the construction of recombinant poxviruses. Gene **65:**123–128.
4. **Choppin, P. W., and A. Scheid.** 1980. The role of viral glycoproteins in adsorption, penetration, and pathogenicity of viruses. Rev. Infect. Dis. **2:**40–61.
5. **Couacy-Hymann, R. F., C. Hurard, J. P. Guillou, G. Libeau, and A. Diallo.** 2002. Rapid and sensitive detection of peste des petits ruminants virus by a polymerase chain reaction assay. J. Virol. Methods **100:**17–25.
6. **Coupar, B. E., M. E. Andrew, G. W. Both, and D. B. Boyle.** 1986. Temporal regulation of influenza haemagglutin expression in vaccinia virus recombinants and effects of the immune response. Eur. J. Immunol. **16:**1479–1487.
7. **Diallo, A., W. P. Taylor, P. C. Lefèvre, and A. Provost.** 1989. Atténuation d'une souche de virus de la peste des petits ruminants: candidat pour un vaccin homologue. Rev. Elev. Med. Vet. Pays Trop. **42:**311–317.
8. **Drillien, R., D. Spehner, A. Kirn, P. Giraudon, R. Buckland, F. Wild, and J. P. Lecocq.** 1988. Protection of mice from fatal measles encephalitis by vaccination with vaccinia virus recombinants encoding either the hemagglutinin or the fusion protein. Proc. Natl. Acad. Sci. USA **85:**1252–1256.
9. **Falkner, F. G., and B. Moss.** 1988. *Escherichia coli gpt* gene provides dominant selection for vaccinia virus open reading frame expression vectors. J. Virol. **62:**1849–1854.
10. **Gershon, P. D., and D. N. Black.** 1989. The nucleotide sequence around the capripoxvirus thymidine kinase gene reveals a gene shared specifically with leporipoxvirus. J. Gen. Virol. **70:**525–533.
11. **Gibbs, P. J. E., W. P. Taylor, M. P. J. Lawman, and J. Bryant.** 1979. Classification of the peste-des-petits-ruminants virus as the fourth member of the genus *Morbillivirus*. Intervirology **11:**268–274.
12. **Kitching, R. P., J. M. Hammond, and W. P. Taylor.** 1987. A single vaccine for the control of capripox infection in sheep and goats. Res. Vet. Sci. **42:**53–60.
13. **Lamb, R. A.** 1993. Paramyxovirus fusion protein: a hypothesis for changes. Virology **197:**1–11.
14. **Lefèvre, P. C., and A. Diallo.** 1990. Peste des petits ruminants. Rev. Sci. Tech. Off. Int. Epizoot. **9:**951–965.
15. **Libeau, G., J. T. Saliki, and A. Diallo.** 1997. Caractérisation d'anticorps monoclonaux dirigés contre les virus de la peste bovine et de la peste des petits ruminants: identifications d'épitopes conservés ou de spécificité stricte sur la nucléoprotéine. Rev. Elev. Méd. Vét. Pays Trop. **50:**109–118.
16. **Merz, D. C., A. Scheid, and P. W. Choppin.** 1980. Importance of antibodies to the fusion glycoprotein of paramyxoviruses in the prevention of spread of infection. J. Exp. Med. **151:**275–288.
17. **Merz, D. C., A. Scheid, and P. W. Choppin.** 1981. Immunological studies of the functions of paramyxovirus glycoproteins. Virology **109:**94–105.
18. **Meyer, G., and A. Diallo.** 1995. The nucleotide sequence of the fusion protein gene of the peste des petits ruminants virus: the long untranslated region in the 5′ end of the F protein gene of morbilliviruses seems to be specific to each virus. Virus Res. **37:**23–38.
19. **Ngichabe, C. K., H. M. Wamwayi, T. Barrett, E. K. Ndungu, D. N. Black, and C. J. Bostock.** 1997. Trial of a capripoxvirus-rinderpest recombinant vaccine in African cattle. Epidemiol. Infect. **118:**63–70.
20. **Ngichabe, C. K., H. M. Wamwayi, E. K. Ndungu, P. K. Mirangi, C. J. Bostock, D. N. Black, and T. Barrett.** 2002. Long term immunity in African cattle vaccinated with a recombinant capripox-rinderpest virus vaccine. Epidemiol. Infect. **128:**343–346.
21. **Ohishi, K., K. Inui, T. Barrett, and K. Yamanouchi.** 2000. Long-term protective immunity to rinderpest in cattle following a single vaccination with a recombinant vaccinia virus expressing the virus haemagglutinin protein. J. Gen. Virol. **81:**1439–1446.
22. **Romero, C. H., T. Barrett, S. A. Evans, R. P. Kitching, P. D. Gershon, C. Bostock, and D. N. Black.** 1993. Single capripoxvirus recombinant vaccine for the protection of cattle against rinderpest and lumpy skin disease. Vaccine **11:**737–742.
23. **Romero, C. H., T. Barrett, R. W. Chamberlain, R. P. Kitching, M. Fleming, and D. N. Black.** 1994. Recombinant capripoxvirus expressing the hemagglutinin protein gene of rinderpest virus: protection of cattle against rinderpest and lumpy skin disease viruses. Virology **204:**425–429.
24. **Romero, C. H., T. Barrett, R. P. Kitching, V. M. Carn, and D. N. Black.** 1994. Protection of cattle against rinderpest and lumpy skin disease with a recombinant capripoxvirus expressing the fusion protein gene of rinderpest virus. Vet. Rec. **135:**152–154.
25. **Stephensen, C. B., J. Welter, S. R. Thaker, J. Taylor, J. Tartaglia, and E. Paoletti.** 1997. Canine distemper virus (CDV) infection of ferrets as a model for testing *Morbillivirus* vaccine strategies: NYVAC- and ALVAC-based CDV recombinants protect against symptomatic infection. J. Virol. **71:**1506–1513.
26. **Taylor, J., R. Weinberg, J. Tartaglia, C. Richardson, G. Alkhatib, D. Briedis, M. Appel, E. Norton, and E. Paoletti.** 1992. Nonreplicating viral vectors as potential vaccines: recombinant canarypox virus expressing measles virus fusion (F) and hemagglutinin (HA) glycoproteins. Virology **187:**321–328.
27. **Tsukiyama, T., Y. Yoshikawa, H. Kumata, H. Imaoka, K. Asano, S. Funahashi, T. Maruyama, H. Shida, M. Sugimoto, and K. Yamanouchi.** 1989. Development of heat-stable recombinant rinderpest vaccine. Arch. Virol. **107:**225–235.
28. **Wild, F., P. Giraudon, D. Spehner, R. Drillien, and J. P. Lecocq.** 1990. Fowlpox virus recombinant encoding the measles virus fusion protein: protection of mice against fatal measles encephalitis. Vaccine **8:**441–442.
29. **Wild, T. F., A. Bernard, D. Spehner, D. Villeval, and R. Drillien.** 1993. Vaccination of mice against canine distemper virus-induced encephalitis with vaccinia virus recombinants encoding measles or canine distemper virus antigens. Vaccine **11:**438–444.
30. **Yilma, T., D. Hsu, L. Jones, S. Owens, M. Grubman, C. Mebus, M. Yamanaka, and B. Dale.** 1988. Protection of cattle against rinderpest with vaccinia virus recombinants expressing the HA or F gene. Science **242:**1058–1061.

HPPRV/Capripox recombinant construction

This part of the work was published in a condensed form in the proceedings of New-York Academy of Sciences, reproduced here below. A complete manuscript was written thereafter and is included in the next pages. Submitted to the review Vaccine, this manuscript has been rejected. Although both reviewers have considered that the data were valid, one of them stated that the paper was too thin for publication in vaccine. Therefore, it is intended to submit this work to another review, possibly enriched with the results on the efficacy of different promoters which are detailed in the next section.

Nevertheless, this work shows that the HPPRV recombinant capripoxvirus, with doses as low as 10 $TCID_{50}$, can protect goats against PPR disease. All vaccinated goats survived while unvaccinated goats died. However, only two out of three goats vaccinated with the lower dose (0.1 $TCID_{50}$) did not show any clinical sign after PPRV challenge while the third one showed an increase of its body temperature (Figure 3 in manuscript 3). On the other hand, all goats vaccinated with the two highest doses did not have detectable amount of PPRV RNA in their peripheral blood mononuclear cells (PBMC, see details in Table 1 of manuscript 3). Nonetheless, goats vaccinated with 10 or 0.1 $TCID_{50}$, although clinically protected, have PPRV RNA in their PBMC, thus illustrating they were infected by the challenge virus. Since all animals vaccinated with the two highest doses of the recombinant had already serum antibodies two weeks post-vaccination, we were not able to measure a boosting effect after PPRV challenge. However, this does not mean that the recombinant capripoxvirus did induce a sterilizing immunity, preventing the replication of PPRV. In fact, like for the FPPRV recombinant capripoxvirus, this recombinant probably needs improvement of its intrinsic immunogenic capabilities. This was attempted later on by trying to increase the level of antigen expression with different promoters (see next section).

Article 2

Goat Immune Response to Capripox Vaccine Expressing the Hemagglutinin Protein of Peste des Petits Ruminants

A. DIALLO,[a] C. MINET,[a] G. BERHE,[a] C. LE GOFF,[a] D.N. BLACK,[b] M. FLEMING,[b] T. BARRETT,[b] C. GRILLET,[a] AND G. LIBEAU[a]

[a]*Cirad, Programme Santé Animale, Campus International de Baillarguet, 34398 Montpellier Cedex 05, France*

[b]*Institute of Animal Health, Pirbright Laboratory, Pirbright, Surrey, GU 24 ONF, UK*

ABSTRACT: **Sheep-pox and capripox are contagious diseases of domestic small ruminants for which the causal agent is a poxvirus classified into the *Capripoxvirus* genus. Viruses of this group have a host range specific to sheep, goats, cattle, and possibly buffalo. Thus, they are clearly indicated as vectors for the development of recombinant vaccines for peste des petits ruminants (PPR). Here we report the immune response of goats inoculated with a recombinant capripox-PPR hemagglutinin.**

KEYWORDS: **capripox vaccine; hemagglutinin protein; immune response; peste des petits ruminants**

INTRODUCTION

Sheep-pox and capripox are contagious diseases of domestic small ruminants. They are characterized by fever, lacrimation, and serous nasal discharge, swelling of the eyelids, congestion of mucous membranes, and respiratory distress. These clinical signs are followed by the development of the characteristic skin lesions: macules that evolve into papules, then vesicles or nodules in some cases. The causal agent is a poxvirus classified in the *Capripoxvirus* genus. Viruses of this group have a host range specific to sheep, goats, cattle, and possibly buffalo. Thus, they are strongly indicated for the development of recombinant vaccines for domestic ruminants[2,3] in areas where they are endemic, including Africa, the Middle East, and Asia. In these areas, the most important contagious small ruminant disease is peste des petits ruminants (PPR), a viral disease characterized by erosive stomatitis, catarrhal inflammation of the ocular and nasal mucous membranes, diarrhea, and death in 50–80% of acute cases. The causal agent is a *Morbillivirus* that has two structural proteins inducing a protective immune response, the fusion protein (F) and the viral attachment protein termed the hemagglutinin (H). PPR shares with capripox nearly the same

Address for correspondence: Dr. G. Libeau, Cirad, Programme Santé Animale, TA 30/G, Campus International de Baillarguet, 34398 Montpellier Cedex 05, France. Voice: 33-467-593850; fax: 33-467-593798.
genevieve.libeau@cirad.fr

Ann. N.Y. Acad. Sci. 969: 88–91 (2002).

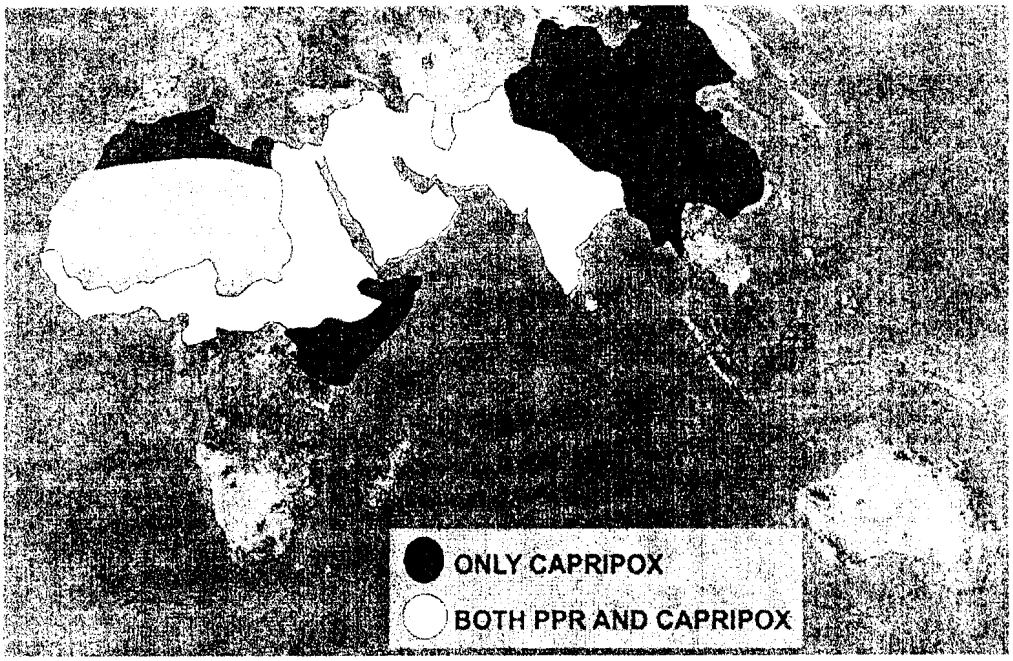


FIGURE 1. Geographical distribution of peste des petit ruminants (PPR) and capripox.

geographic distribution (FIG. 1). The H gene of the PPR virus was inserted into the genome of the attenuated capripox KS-1.[1] The generated recombinant capripox-PPR hemagglutinin can protect goats against the virulent PPR virus. In another study, we demonstrated the dual vaccine property of an attenuated capripox virus expressing the PPR F protein.[4]

MATERIALS AND METHODS

The *Escherichia coli* xanthine-guanine phosphoribosyltransferase gene (*Eco gpt*) was used as a dominant selectable marker for the isolation of the recombinant. Its corresponding DNA and the H PPR cDNA, both under the control of a synthetic promoter, were inserted into the thymidine kinase (TK) gene of the attenuated capripox KS1 genome (FIG. 2), as reported by Romero *et al.*[2,3]

RESULTS AND DISCUSSION

Lamb testis cells (LT) that were infected with one selected recombinant (rec-ca-HPPR) reacted positively from immunofluorescent staining with monoclonal anti-HPPR and FITC-conjugate anti-mouse. This result indicated that the recombinant virus expressed the PPR H protein. Its protective immune response was tested in goats by subcutaneous inoculation. The following doses of the recombinant were tested: 10^5, 10^3, 10, and 0.1 $TCID_{50}$/animal. After a PPR challenge 3 weeks post-

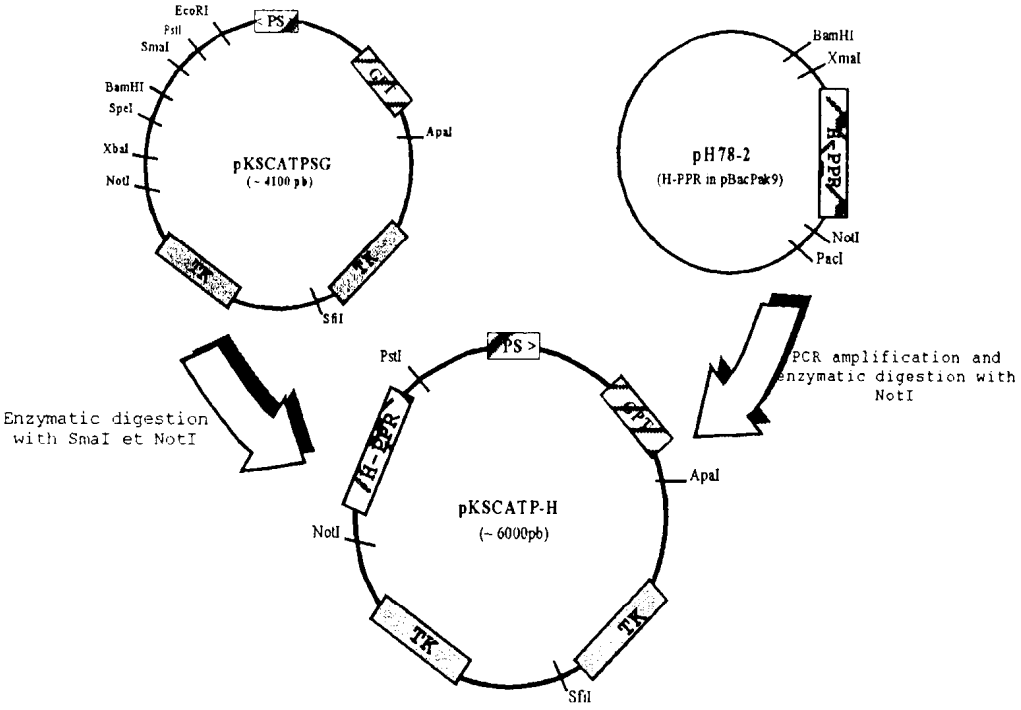


FIGURE 2. Insertion of the H-PPR into the TK gene of the capripox KS1 vaccine strain.

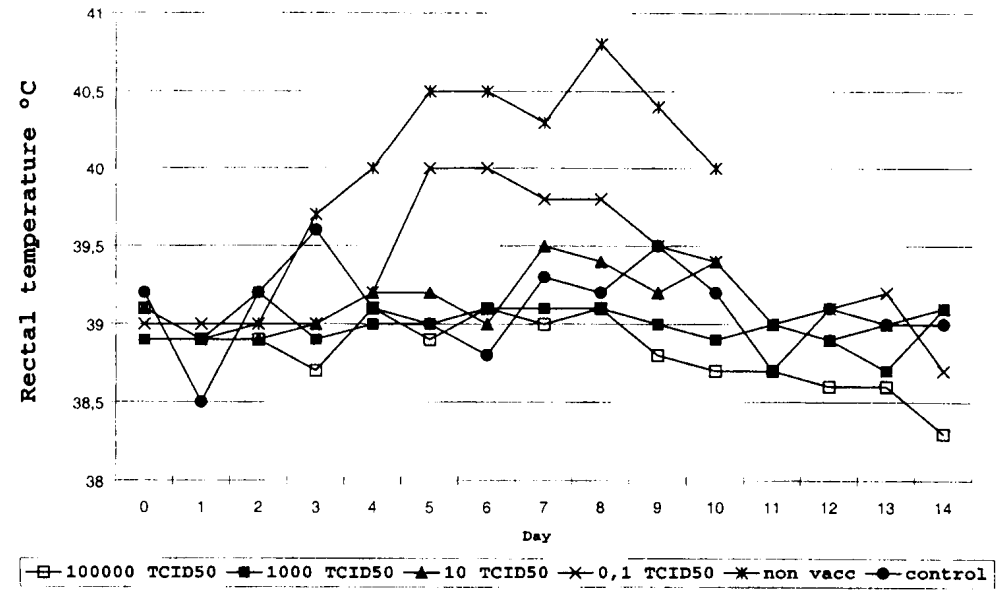


FIGURE 3. Minimum effective vaccine dose. Rectal temperature after PPR challenge.

vaccination, only control animals and those vaccinated with the 0.1 $TCID_{50}$ became sick and died (FIG. 3). These animals showed a dramatic decrease in the number of white blood cells, whereas animals resistant to the challenge did not show a significant change in this biological parameter and also developed PPR neutralizing anti-

bodies (not shown). These results indicate that the recombinant virus re-ca-HPPR, at a dose of at least 10 $TCID_{50}$, can protect goats against virulent PPR virus. This dose is 100 times greater than the capripox-F PPR recombinant[4] that provides a protective immune response at a dose of 0.1 $TCID_{50}$.

REFERENCES

1. KITCHING, R.P., J.M. HAMMOND & W.P. TAYLOR. 1987. A single vaccine for the control of capripox infection in sheep and goats. Res. Vet. Sci. **42:** 53–60.
2. ROMERO, C.H. *et al.* 1993. Single capripox recombinant vaccine for the protection of cattle against rinderpest and lumpy skin disease. Vaccine **11:** 737–742.
3. ROMERO, C.H. *et al.* 1994. A recombinant capripoxvirus expressing the haemagglutinin protein gene of rinderpest virus: protection of cattle against rinderpest and lumpy skin disease viruses. Virology **204:** 425–429.
4. BERHE, G. *et al.* Development of dual recombinant vaccine to protect small ruminants against peste des petits ruminants and capripox infections. Submitted for publication.

Manuscript 3

Construction of a Recombinant Capripox Virus Expressing Peste des Petits Ruminant Virus Haemagglutinin and Evaluation of its Protective Immune Response in Goats.

Berhe[1ab] G., C. Minet[1b], C. Le Goff[1b], T. Barrett[2], P. Gil[1], C. Grillet[1], G. Libeau[1], E. Albina[1] and A. Diallo[1c*]

[1]*EMVT Department-CIRAD, Control of Exotic and Emerging Animal Diseases (UPR15), TA 30/G, Campus International de Baillarguet, 34398 Montpellier Cedex 05, France;*

[2]*Institute for Animal Health, Pirbright Laboratory, Pirbright, Surrey, GU 24 ONF, UK*

[b]: Mr Berhe G. and Ms Minet C. contributed equally to the construction of the recombinant and its testing in animals. Mr Le Goff constructed the transfer vector plasmid.

Present address:

[a] National Veterinary Institute, NVI, Debre Zeit, P.O. BOX 19, Ethiopia

[c]Animal Production Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, IAEA Laboratories, A-2444 Seibersdorf, Austria; tel: (+43-1) 2600 28355; fax: (+43-1) 2600 28222; e-mail: a.diallo@iaea.org

[*] To whom correspondence should be addressed.

SUMMARY

The capripox virus KS1 was used as a vector to express the peste des petits ruminants virus (PPRV) haemagglutinin protein (H) under the control of a synthetic poxvirus promoter. The PPRV recombinant protein produced reacted strongly in an immunofluorescence test using a monoclonal antibody specific to PPRV H protein. Evaluation of the immune response of goats to this recombinant virus showed the production of PPRV neutralizing antibodies in animals that were inoculated with a dose of 10 $TCID_{50}$ or more of the virus. All these goats resisted challenge with a virulent strain of the virus. The minimum protective dose was lower than that obtained with either a capripox or vaccinia-based rinderpest haemagglutinin recombinant vaccine but was 10-fold higher than that for a recombinant capripox virus expressing the PPRV fusion protein (29)**.**

Keywords: recombinant vaccine; capripox vector; PPR, morbillivirus

Short title: Capripox-based PPRV haemagglutinin recombinant vaccine

1. INTRODUCTION

Peste des petits ruminants (PPR) is a highly contagious disease of small ruminants and is one of a group of animal diseases for which outbreaks must to be reported to the World Organisation for Animal Health (Office International des Epizooties: OIE). It is a disease which constitutes the main constraint to the increased production of sheep and goats in many African countries south of the Sahara, in the Middle East, in Turkey and in South Asia (1; 2; 3). Thus its control is a major concern for poverty alleviation in many parts of Africa and South Asia (4). A live-attenuated vaccine derived from the PPRV Nigeria 75/1 strain by serial passage on Vero cells is currently used to control this disease in some areas (5). For use in countries with hot climates, its thermostability has been improved by dehydration of the virus suspension in the presence of trehalose (6). A recent investigation revealed that, even though many countries consider PPR to be an economically important disease, most implement vaccination campaigns only when outbreaks are detected and with the objective of limiting the spread of disease (7). One of the factors which may explain why so few countries undertake systematic vaccination campaigns to control PPR is certainly the high cost of the logistics needed to vaccinate small ruminants whose populations are scattered widely over large areas. A way to increase the cost effectiveness of vaccination is to attempt to control more than one economically significant disease of sheep and goats in the same operation using multivalent vaccines. Capripox virus appears to be an ideal vector for the development of such vaccines for ruminants (8). The causal agent of peste des petits ruminants is a virus, the *Peste des petits ruminants virus* (PPRV), which is classified in the *Morbillivirus genus* within the family *Paramyxoviridae* (9). This genus includes other closely related animal and human pathogens: rinderpest virus (many species of

ruminants), measles virus (humans), canine distemper virus (carnivores), phocid distemper virus (seals) and the cetacean morbilliviruses (whales, dolphins and porpoises). As is the case for other paramyxoviruses, PPRV is an enveloped non-segmented negative sense single-stranded RNA virus with two external glycoproteins, the fusion (F) and haemagglutinin (H) proteins. These proteins are indispensable for infection of host cells by the virus and following attachment and fusion of the virus envelope with the host cell the viral nucleocapsid is delivered into the cytoplasm where virus replication takes place. The H protein mediates attachment of the virus to the host cell membrane via its interaction with the cellular receptor(s) while the F protein enables the virus to penetrate into the cell by effecting fusion of its membrane with that of its target cell. With some paramyxoviruses, including PPRV, the F protein alone cannot effect cell fusion and it needs the cooperation of the H protein (10; 11; 12; 13; 14). Moreover, in the case of PPRV and rinderpest virus (RPV), two closely related ruminant viruses, it has been demonstrated that the H protein gene of one virus cannot be replaced by that of the other to allow effective activity of the F protein and virus viability (15), therefore these two proteins are absolutely required for the initiation and propagation of the viral infection. They are also the two viral proteins which have been shown to provide protective immunity against morbillivirus infections (16; 17; 18; 19; 20) and both F and H have been used to develop effective recombinant morbilliviruses vaccines (21; 22; 23; 24; 25; 26; 27; 28). Most neutralizing antibodies produced in an infected animal are directed against the H protein, the protective property of the F protein being mostly through cell-mediated immunity (18). In a previous publication, we reported on the insertion of the PPRV F gene into the capripox virus genome and on the efficacy of this recombinant virus as a dual vaccine to protect small ruminants against both capripox

and PPR infections (29). Here we report on the construction of a recombinant capripox virus expressing the PPRV H protein and the results of a preliminary trial in goats as a vaccine against PPR.

2-MATERIALS AND METHODS

2-1. Cells and viruses.

Primary lamb testis (LT) and Vero cells were used to propagate capripoxviruses and PPRV respectively. LT cells were grown in Dubelco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) and antibiotics. For Vero cells, the growth medium used was Eagle's minimal essential medium (MEM) containing 10% FCS and antibiotics. The attenuated capripoxvirus Kenya sheep-1 (KS-1) vaccine was used as the vector virus (30). The virulent PPRV Guinée Bissau /89, a virus strain isolated from a goat during a PPR outbreak in Guinée Bissau in 1989 (Diallo A., unpublished data), was used as the challenge virus in the vaccine trial (29).

2-2 Construction of the recombinant

The transfer vector, pKSCATPSG (figure 1), was constructed in our laboratory. It is a pBluescript II KS plasmid into which was inserted a back-to-back poxvirus synthetic double early/late promoter (see sequence in figure 1a). This double synthetic promoter (PS), a gift from Dr R. Drillien (Strasbourg, France), was proved to give strong protein expression in the vaccinia virus vector system (31). To enable homologous recombination between the transfer vector and the capripox viral genome, a fragment of

the capripox virus thymidine kinase gene (TK) was inserted at each end of the plasmid polylinker. They were 288 and 396 base pairs (bp) long and are indicated in figure 1b by TK_L for TK left, and TK_R for TK right, respectively. The plasmid also contains the *E. coli* xanthine-guanine-phosphoribosyltransferase gene (*Eco gpt*) under the control of one of the synthetic promoters. *Eco gpt* was used as the dominant selectable marker to isolate the recombinants (32; 33). A number of unique restriction enzyme sites used for the cloning of foreign inserts are located downstream of the second promoter. As indicated in figure 1, cDNA corresponding to the haemagglutinin gene of the PPRV vaccine strain Nigeria 75/1 (accession number Z81358 or X74443) was inserted into pKSCATPSG between the *Sma*1 and *Not*1 restriction sites. The final construct, plasmid pKSCATP-H, was used to generate the recombinant virus with the capripox virus KS-1 in LT cells as described previously (29). The virus obtained following transfection of capripoxvirus KS1-infected cells with the transfer plasmid pKSCATP-H was used to select the recombinants.

2-3. Selection and characterization of the recombinants clones

The method described by Romero *et al.* (26) and modified by Berhe *et al.*, (29) was used to select the recombinants. Briefly, this was done by limiting dilution and plaque selection in the presence of selection medium which contains mycophenolic acid (MPA), xanthine and hypoxanthine. Before each dilution, the viral suspension was sonicated for 30 sec in a water bath sonicator. The presence of the recombinant in viral suspensions and its purity were checked by PCR with both capripox virus TK and PPRV H gene-specific primers. The capripox primers were designed in the TK gene of the capripox

virus KS-1: CPTK7 ACTTATCAGATTTTGTTACGACATT and CPTK8 CGATGAGTTCTATTTCCTTTTCTTTAG. They bind to the transfer vector in the TK_L and TK_R fragments respectively. HP46 CGCACAAAGGGAAAGGATCA and HP47 CTGATGCCGACTTCATCACC were the primers used to amplify a fragment of the PPRV H gene. The conditions of the PCR were those reported previously (29).

2-4 Detection of the recombinant H protein

The expression of the PPRV H protein by the recombinant capripox-PPRVH virus (recCapPPR/H) selected in the LT cells was confirmed by immunofluorescence as described previously (29). The PPRV H protein-specific monoclonal antibody used was the Mab B2G3 which was described previously (34).

2-5. Vaccination and Challenge of goats

Fourteen British goats of mixed breed were purchased and housed in 6 different groups in the high-security animal accommodation at the Institute for Animal Health, Pirbright Laboratory. Groups I, II, III, and IV were each composed of 3 animals that were inoculated by subcutaneous injection with 10^5, $10^{3,}$ 10, and 10^{-1} $TCID_{50}$, respectively, of the recombinant virus recCapPPR/H. Group V, composed of 2 animals, did not receive any injection. The animals were examined daily for clinical signs of infection. One month later all animals, including the controls of group V, were challenged with the virulent PPR virus Guinée Bissau/89 strain at 10^4 $TCID_{50}$/animal by subcutaneous injection. They were observed daily for clinical signs of PPR infection for two weeks

following challenge. Animals were bled every week for the collection of serum and every 2 days for the preparation of peripheral blood leucocytes (PBL) which were purified on Ficoll-Paque gradients according to the manufacturer's instructions (Amersham Biosciences).

2-6. Antibody Assay

The sera were tested for the presence of antibodies against PPR using both the H-based cELISA as described by Anderson et al. (35) and the virus neutralisation test (VNT). The latter was performed in the 96-microwell plates. Briefly, 100 $TCID_{50}$ of PPRV vaccine strain Nigeria 75/1 (5) in 100µl of cell culture medium were mixed with 100µl of the diluted test serum in the well. Twofold serial dilutions were made from a 1/5 pre-dilution and before use in VNT, the sera were heat-inactivated at 56 °C for 30 min. After 1 hour of incubation at 37°C, Vero cells (2×10^4) in 50 µl of cell culture medium were added. The plates were incubated at 37 °C and read at 10, 12 and 14 days for the detection of virus cytopathic effect (cpe). The 14 day reading was used to determine the titre. A serum was considered positive for PPRV neutralizing antibodies if no cytopathic effect was detected at dilutions of 1/10 or higher.

2-7. Detection of the PPR challenge virus

Total RNAs were extracted from PBL that were collected from the animals at various times after challenge. The extractions were made using RNeasy kit according to the manufacturer's instructions (Qiagen). The RNAs were then submitted to RT-PCR tests as described previously to detect the N gene-specific RNA of the challenge virus (29; 36).

3. RESULTS

3-1. Isolation of the recombinant

The methodology that proved to be quick and efficient for the selection of pure capripox-based recombinant viruses, as outlined in Materials and Methods, that is limiting dilution of sonicated virus suspensions and verification of virus purity by PCR was followed (29). For this we used primers HP46 and HP 47 to amplify a fragment of 312 nucleotides on the PPRV H gene. The same sample was tested using another pair of primers specific to the capripox virus TK gene: primers CPTK7 and CPTK8 binding to TK_L and $TK_{R,}$ respectively. In the absence of a foreign gene insert in the TK gene they allowed the amplification of a fragment of about 600 nucleotides from capripox genome. When recombination occurred the genome of the virus would contain both the PPRV H and the selection marker genes and the expected size of the amplified target would then be about 2500 nucleotides. The conditions of elongation in the PCR reactions did not allow the amplification of targets greater than about 1000 nucleotides (29), thus if a virus suspension contained only the pure recombinant no DNA was amplified with primers CPTK7 and CPTK8, while the PCR would be positive with the HP46 and HP47 primer set. In the case of there being a mixture of recombinant and parental viruses, both sets of primers would give positive PCR products. After two limiting dilution selections using this technique, the recombinant viral suspension was plaque purified under low melting point agarose using. Five clones positive by PCR with the HP46-HP47 primers but negative with the CPTK7-CPTK8 primers were selected. One clone, recCapPPR/H, was used as a pure recombinant capripox virus-HPPR and stocks prepared for subsequent use.

The expression of the recombinant protein by this virus was confirmed by immunofluorescence using a PPRV H-specific monoclonal antibody (B23G) according to the method of Berhe *et al.,* (29). As can be seen in figure 2, fluorescence was detected in cells infected with the recombinant virus but not in uninfected cells.

3-2. Evaluation of the protective capacity of the recombinant recCapPPR/H virus in goats.

To assess the ability of the recombinant recCapPPR/H virus to protect animals against PPR infection, twelve goats housed in groups of three were vaccinated subcutaneously with virus doses ranging from 10^5 to 10^{-1} $TCID_{50}$/animal as indicated in Materials and Methods. Two goats to be used as challenge controls did not receive any injection of the recombinant. During the one month of clinical observation following vaccination, all animals remained healthy. After this period, the vaccinated animals and the two controls which did not receive any treatment were challenged with 10^4 $TCID_{50}$ of PPRV Guinée Bissau 89/1. All goats in groups I, II, III and two of group IV (goats UD38 and 39) showed no clinical signs of PPRV infection. Their rectal temperatures remained normal during the two weeks of observation although animals UD 36 and UD 37, which were inoculated with 10 $TCID_{50}$ of the recombinant virus, had rectal temperatures of 39.7 to 39.9 °C for one or two days (figure 3). One goat, UF3, which received 10^{-1} $TCID_{50}$ of the recombinant virus developed fever and leucopoenia (see below) for about one week but recovered. The two challenged control animals had high temperatures and other PPR clinical signs which were so severe that they were humanely euthanized at 11 and 12 days post infection. PPRV is a lymphotropic virus, as are all morbilliviruses, and leucopoenia is one of the clinical features of the disease. This parameter was evaluated in the present

study. At 0, 2, 4, 7, 9, 10 and 11 days post challenge, PBL were collected from all animals. The cells were counted and any reduction ≥50% in the number in comparison to that of the day of challenge was considered as a sign of significant leucopoenia. Based on that criterion, no abnormality was observed with animals vaccinated with 10^5-10^3 $TCID_{50}$ of the recombinant virus. All other animals develop leucopoenia lasting for at least one day following the challenge (data not shown). Growth of the PPRV challenge virus in the vaccinated animals was monitored by the detection of virus-specific RNA sequences by a PPRV N gene-based RT-PCR on total RNA extracted from the PBL. As can been seen in table 1, the test was negative with the PPRV specific primers NP3-NP4 for the samples collected from challenged animals of group I and II and also with one goat of the group III, UD 36. For the two other animals in group III, the RT-PCR was positive as for the control challenged goats. This indicated that 10 $TCID_{50}$ of the recombinant recCapPPR/H is not sufficient to prevent replication of the challenge virus to a level detectable by RT-PCR, even in absence of an overt disease.

3-3. Serological response

The results of assays to measure the serological responses in animals after vaccination with the recombinant virus but before challenge are summarised in table 2. Two different techniques, the VNT and the H protein-based competitive ELISA (cELISA), were used for the detection of PPRV antibodies. The positive / negative cut-off value in the cELISA was taken as 50% inhibition of the binding of the monoclonal antibody while for the VNT a serum was considered to have antibodies against PPRV if it neutralised the virus at a dilution of at least 1/10. Both tests were positive one to two weeks after vaccination for the goats inoculated with the recombinant virus at doses ranging from 10^5 to 10

$TCID_{50}$. However, one animal of the group that received a vaccine dose of 10 $TCID_{50}$, goat UD 37, showed a very weak antibody response only detected by cELISA in serum collected at 2 weeks post vaccination. All these animals were protected from PPR disease following virulent challenge. Within the group that received a dose of 10^{-1} $TCID_{50}$, one animal, UD 38, showed a very weak positive PPRV antibody response, again only detected by cELISA. Serological tests remained negative for the two other goats. In the same group, only goat UF3 developed a pyrexic response which lasted for about one week following challenge.

3. DISCUSSION

In a previous study, we demonstrated the potential of a recombinant capripox virus expressing the PPRV F protein as an effective dual vaccine to protect goats against both capripox and PPRV infections. Here we report on the protective properties of another capripox-based PPRV recombinant vaccine. This new recombinant carries the second PPRV glycoprotein gene, that encoding the H protein. Three plasmids were used to construct the transfer vector containing the cDNA corresponding to the F gene (29) but the construction of the new transfer vector carrying the PPRV H cDNA was much simplified by the use of a plasmid containing a double poxvirus promoter, the selection marker gene and sequences of the capripox TK gene to enable homologous recombination to take place. The recombinant virus proved effective in protecting goats against PPR disease at a minimal dose of 10 $TCID_{50}$. With the 10^{-1} $TCID_{50}$ virus dose no animal died following virulent challenge but one animal got a high fever which lasted for about one week. Ten $TCID_{50}$ of virus which was found to protect animal against overt disease on challenge is a dose far lower than that reported for poxvirus-based rinderpest

H recombinants. With the recombinant vaccinia-RPV H, a minimum dose of $10^4 – 10^5$ PFU is required to achieve complete protection of cattle against rinderpest (37) while with the capripox-based RPV H recombinant, $2.4x10^3$ PFU were efficacious in protecting cattle against virulent rinderpest challenge (27). The disparity between these reports and the results from the present study may be explained by the differences in species and their response to the vector virus or by differences in the promoters that were used: promoter p7.5 for the vaccinia-RPV H, promoter p11 for the capripox-RPV H recombinant and a synthetic early/late promoter for the capripox-PPRV H. Another possible reason could be the degree of purity of the recombinants used. The purity of the rinderpest recombinants was not checked by the very sensitive PCR test, as was the case in the current study and the presence of some parental vector virus in the recombinant vaccine cannot be ruled out. If 10 $TCID_{50}$ of recCapPPR/H virus is the minimal protective dose of goats against PPRV infection, this would represent a higher dose than the 0.1 PFU necessary for the capripox PPRV F recombinant (29). Both PPRV recombinants were selected using the same technique and the stocks were shown to be free of parental virus. The difference between these two capripox-based PPR recombinant vaccines may reflect either a better immunoprotective quality of the fusion protein compared to that of the haemagglutinin or a difference in promoter efficiency. While expression of the fusion protein is driven by the vaccinia virus promoter p7.5, expression of the H protein gene was placed under the control of a synthetic early/late promoter capripox. Work to compare the efficacy of different poxvirus promoters for the induction of protective immunity in goats against PPR disease is in progress.

The results obtained with the capripox-based PPRV F and H recombinants are similar to those that have been reported for rinderpest recombinant vaccines and are consistent to

the observation that most of morbillivirus neutralising antibodies are directed against the haemagglutinin (18; 26; 27). However, there are indications that a humoral response alone is not the only basis of the protective properties of the haemagglutinin and that this protein may also induce a cell-mediated immune response (38; 39). This possibility may explain why goat UD 39, which did not show any detectable antibody response to the capripox-PPRV H recombinant at the dose of 0.1 $TCID_{50}$, did not show a clinical response to the challenge virus although it replicated for many days in the animal as evidenced by the detection of virus-specific RNA in the PBL. Two T cell epitopes have been mapped on the PPRV H protein (39) and a baculovirus-expressed rinderpest H protein is only protective if delivered in a suitable T-cell inducing adjuvant such as an ISCOM (38). A similar report on the protection of seals from phocine distemper virus (PDV) showed that inactivated canine distemper virus incorporated into ISCOMs was able to protect seals from the phocine distemper virus (40). Therefore the best way to deliver a PPRV single gene-based vaccine is through a system which can trigger both humoral and cellular immune responses, as happens with a live virus vector. For this purpose the capripox vector has the advantage of being very specific to cattle, sheep and goats without the adverse effect of vaccinia virus on the human population. Moreover, the capripox-based PPRV recombinant vaccine can be used to protect sheep and goat against both capripox and PPRV, two diseases which have a highly negative impact on small ruminant production in many developing countries (29).

ACKNOWLEDGMENTS:

We thank Dr R. Drillien (INSERM, Strasbourg, France) for having provided us with the poxvirus synthetic promoter. This work was supported by a grant from the Ministère des

Affaires Etrangères (projet RAVIRA), France. B.G was supported by a fellowship from the Ministère des Affaires Etrangères, France. The work carried out at the Pirbright Laboratory was supported by the Biotechnology and Biological Research Council, United Kingdom.

REFERENCES

[1] Lefèvre, P.C. and Diallo, A. Peste des Petits Ruminants. Rev. Sci. Tech.Off. Int. Epiz. 1990; 9 : 951-65

[2] Ozkul A, Akca Y, Alkan F, Barrett T, Karaoglu T, Dagalp SB, Anderson J, Yesilbag K, Cokcaliskan C, Gencay A, Burgu I. Prevalence, distribution, and host range of Peste des petits ruminants virus, Turkey. Emerg Infect Dis. 2002; 8:708-12

[3] Elzein EM, Housawi FM, Bashareek Y, Gameel AA, Al-Afaleq AI, Anderson E. Severe PPR infection in gazelles kept under semi-free range conditions. J Vet Med B Infect Dis Vet Public Health. 2004; 51(2):68-71.

[4] Perry, B.D., Randolph, T.F., McDermott, J.J., Sones, K.R. and Thornton, P.K. Investing in animal health research to alleviate poverty. *ILRI (International Livestock Research Institute), Nairobi, Kenya,* 2002, 148 pp

[5] Diallo A., Taylor W.P., Lefèvre P.C., Provost A. Atténuation d'une souche de virus de la peste des petits ruminants: candidat pour un vaccin homologue. Rev. Elev. Méd. vét. Pays Trop. 1989; 42: 311-17

[6] Worwall E. E., Litamoi J. K., Seck B. M. and Ayelet G. *Xerovac*: an ultra rapid method for the dehydration and preservation of live attenuated Rinderpest and Peste des Petits ruminants vaccines. Vaccine 2001; 19: 834–39.

[7] Diallo (A). Control of Peste des Petits ruminants: vaccination for the control of Peste des Petits Ruminants. *Dev Biol. Basel*, 2005; 119: 93-98.

[8] Black, D.N. Capripox-based multivaccines. *Dev Biol. Basel* 1995; 84: 179-82.

[9] Gibbs, P.J.E., Taylor, W.P., Lawman, M.P.J. & Bryant, J. Classification of the peste-des-petits-ruminants virus as the fourth member of the genus *Morbillivirus*. Intervirology 1979; 11: 268-74

[10] Wild, T. F., E. Malvoisin, and R. Buckland. Measles virus: both the hemagglutinin and fusion glycoproteins are required for fusion. J. Gen. Virol. 1991; 72: 439-42

[11] Ebata S. N., M.-J. Cote, C. Y. Kang, and K. Dimock. The fusion and hemagglutinin-neuraminidase glycoproteins of human parainfluenza virus 3 are both required for fusion. Virology 1991; 183**:** 437-41

[12] Hu X., R. Ray, and R. W. Compans. Functional interactions between the fusion protein and hemagglutinin-neuraminidase of human parainfluenza viruses. J. Virol. 1992; 66:1528-34

[13] Cattaneo R., and J. K. Rose. Cell fusion by the envelope glycoproteins of persistent measles viruses which caused lethal human brain disease. J. Virol. 1993; 67**:** 1493-1502

[14] Heminway, B. R., Y. Yu, and M. S. Galinski. Paramyxovirus mediated cell fusion requires coexpression of both the fusion and hemagglutinin-neuraminidase glycoproteins. Virus Res. 1994; 31:1-16

[15] Das S. C., Michael D. Baron, and Thomas Barrett. Recovery and Characterization of a Chimeric Rinderpest Virus with the Glycoproteins of Peste-des-Petits-Ruminants Virus: Homologous F and H proteins are required for Virus Viability J. Virol. 2000; 74: 9039-47

[16] Choppin P.W. and Scheid, A. The role of viral glycoproteins in adsorption, penetration, and pathogenicity of viruses. Rev. Infect. Dis. 1980; 2: 40-61

[17] Merz, D.C., Scheid A., Choppin P.W. Immunological studies of the functions of Paramyxovirus glycoproteins. Virology 1981; 109: 94-105.

[18] Giraudon P. and Wild, T. F. Correlation between epitopes on hemagglutinin of measles virus and biological activities: passive protection by monoclonal antibodies is related to their hemagglutination inhibiting activity. *Virology* 1985; 144: 46-58.

[19] Malvoisin E. and Wild, F. Contribution of measles virus fusion protein in protective immunity: anti-F monoclonal antibodies neutralize virus infectivity and protect mice against challenge. J. Virol. 1990; 64**:**5160-62

[20] Fournier P., Brons, N. H. C., Berbers, G. A. M., Wiesmüller, K.-H., Fleckenstein, B. T., Schneider, F., Jung, G. & Muller, C. P. Antibodies to a new linear site at the topographical or functional interface between the haemagglutinin and fusion proteins protect against measles encephalitis. J. Gen. Virol. 1997; 78: 1295-1302.

[21] Drillien R, Spehner D, Kirn A, Giraudon P, Buckland R, Wild F, Lecocq JP. Protection of mice from fatal measles encephalitis by vaccination with vaccinia virus recombinants encoding either the hemagglutinin or the fusion protein. Proc. Natl. Acad. Sci. USA 1988; 85: 1252-56.

[22] Tsukiyama T., Yoshikawa Y., Kamata H., Imaoka H., Asano K., Funahashi S., Maruyama T., Shida H., Sugimoto M. and Yamanouchi K. Development of heat-stable recombinant rinderpest vaccine. Arch. Virol. 1989; 107: 225-35

[23] Barrett T, Belsham GJ, Subbarao SM, Evans SA. Immunization with a vaccinia recombinant expressing the F protein protects rabbits from challenge with a lethal dose of rinderpest virus. Virology 1989; 170: 11-18

[24] Wild F, Giraudon P, Spehner D, Drillien R, Lecocq JP. Fowlpox virus recombinant encoding the measles virus fusion protein: protection of mice against fatal measles encephalitis. Vaccine 1990; 8: 441-42

[25] Taylor J, Weinberg R, Tartaglia J, Richardson C, Alkhatib G, Briedis D, Appel M, Norton E, Paoletti E**.** Non replicating viral vectors as potential vaccines: recombinant canarypox virus expressing measles virus fusion (F) and hemagglutinin (HA) glycoproteins. Virology 1992; 187: 321-28

[26] Romero CH, Barrett T, Evans SA, Kitching RP, Gershon PD, Bostock C, Black DN. 1993. Single capripoxvirus recombinant vaccine for the protection of cattle against rinderpest and lumpy skin disease. 1992; Vaccine 11: 737-42

[27] Romero CH, Barrett T, Chamberlain RW, Kitching RP, Fleming M, Black DN. Recombinant capripoxvirus expressing the hemagglutinin protein gene of rinderpest virus: protection of cattle against rinderpest and lumpy skin disease viruses. Virology 1994; 204: 425-[28] Stephensen CB, Welter J, Thaker SR, Taylor J, Tartaglia J, Paoletti E. Canine distemper virus (CDV) infection of ferrets as a model for testing Morbillivirus vaccine strategies: NYVAC- and ALVAC-based CDV recombinants protect against symptomatic infection. J. Virol. 1997; 71: 1506-13.

[29] Berhe G, Minet C, Le Goff C, Barrett T, Ngangnou A, Grillet C, Libeau G, Fleming M, Black DN, Diallo A. Development of a dual recombinant vaccine to protect small ruminants against peste-des-petits-ruminants virus and capripoxvirus infections**.** J. Virol., 2003; 77**:** 1571-77

[30] Kitching, R.P., Hammond,J.M. and Taylor, W.P. A single vaccine for the control of capripox infection in sheep and goats. Res. Vet. Sci. 1987; 42: 53-60

[31] Chakrabarti S, Sisler JR, Moss B. Compact, synthetic, vaccinia virus early/late promoter for protein expression. Biotechniques, 1997, 23:1094-7

[32] Boyle, D.B. and Coupar, B.E.H. A dominant selectable marker for the construction of recombinant poxviruses. Gene 1988; 65: 123-28

[33] Falkner, F.G. and Moss, B. Escherichia Coli gpt gene provides dominant selection for vaccinia virus open reading frame expression vectors. J. Virol. 1988; 62: 1849-54

[34] Saliki JT, Libeau G, House JA, Mebus CA, Dubovi EJ. Monoclonal antibody-based blocking enzyme-linked immunosorbent assay for specific detection and titration of peste-des-petits-ruminants virus antibody in caprine and ovine sera. J Clin Microbiol., 1993, 31:1075–1082.

[35] Anderson J. and McKay J.A. The detection of antibodies against peste des petits ruminants virus in cattle, sheep and goats and the possible implications to rinderpest control programmes. Epidemiol. Infect., 1994; 112: 225-31

[36] Couacy-Hymann, Roger F., Hurard C., Guillou J.P., Libeau G., and Diallo A. Rapid and sensitive detection of peste des petits ruminants virus by a polymerase chain reaction assay. J. Virol. Meth., 2002; 100: 17-25

[37] Yamanouchi, K., Inui, K., Sugimoto, M., Asano, K., Nishimaki, F., Kitching, R.P., Takamatsu, H. and Barrett, T. Immunisation of cattle with a recombinant vaccinia vector expressing the haemagglutinin gene of rinderpest virus, Vet. Rec., 1993; 132: 152-56

[38] Kamata H., Kazue Ohishi, Ellena Hulskotte, Albert D. M. E. Osterhaus, Kenjiro Inui, M. S. Shaila, Kazuya Yamanouchi and Thomas Barrett. Rinderpest virus (RPV) ISCOM vaccine induces protection in cattle against virulent RPV challenge. Vaccine, 2001; 19: 3355-59

[39] G. Sinnathamby, G. J. Renukaradhya, M. Rajasekhar, R. Nayak and M. S. Shaila. Immune responses in goats to recombinant hemagglutinin-neuraminidase glycoprotein of *Peste des petits ruminants* virus: identification of a T cell determinant. Vaccine, 2001; 19: 4816-23

[40] Visser, I. K. G., Vedder, E.J., van de Bildt, M. W. G., Orvell, C., Barrett, T. and Osterhaus, A. D. M. E. Canine distemper virus ISCOMs induce protection in harbour seals against phocid distemper but still allow subsequent infection with phocid distemper virus-1. Vaccine, 1992, 10: 435-438

FIGURE LEGENDS

Figure 1. (a) Sequence of the synthetic early/late double promoter (31). Nucleotides in italic are elements required for the sequence to act as early or late promoter. (b) Strategy for construction of the transfer vector. Plasmid pKSCATPSG was derived from pBluescript into which a back-to-back double poxvirus synthetic early/late promoter, the Eco gpt gene and two fragments of the capripox TK gene, were inserted. The cDNA corresponding to the coding sequence of PPR H gene was amplified with the proofreading enzyme *Pfu* from plasmid pH78-2 using two primers, the HP78 (ACCATGTCCGCACAAAGGGAAAGG) which starts at the ATG of the H gene ORF and the Bac Hist (ACAACGCACAGAATCTAGCG) binding to the original plasmid outside the H gene. The amplified product which is blunt at both ends was digested with *Not* 1 and ligated into the plasmid double digested with the restriction enzymes *Not*1 and *Sma*1. The sequence of the insert was checked before further use.

Figure 2. Detection of the PPRV H protein by immunofluorescence staining. LT cells were infected with recCapPPR/H virus (a) or capripox virus KS1 (b). At two days post-infection, they were stained for indirect immunofluorescence with a PPRV-specific anti-H monoclonal antibody as previously described (29).

Figure 3. Daily rectal temperatures of vaccinated and control goats after challenge with the virulent PPR Guinée Bissau/ 89 strain. The different doses of the recombinant recCapPPR/H virus used are indicated in each panel and the days post challenge on the X-axis.

Table 1: Detection of PPRV N gene-specific RNA in PBL collected from goats after challenge with the virulent PPRV Guinée
The PCR test was performed as described by Berhe et al (29) and Couacy et al. (34). The β actin-specific primers were used to amplify the β actin mRNA as a control for the quality of the extracted RNA

	Dose of virus	10^5 pfu			10^3 pfu			10 pfu			0.1 pfu			control challenge	
	n° of goat	UD32	UD33	UD41	UD34	UD35	UF1	UD36	UD37	UF2	UD38	UD39	UF3	UD40	UF5
Primers	day post challenge														
βActin	D 0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NP3/Np4		-	-	-	-	-	-	-	-	-	-	-	-	-	-
βActin	D 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NP3/Np4		-	-	-	-	-	-	-	-	-	-	-	-	-	-
βActin	D4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NP3/Np4		-	-	-	-	-	-	**-**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
βActin	D 7	+	+	+	+	+	+	+	+	nd	+	+	+	+	+
NP3/Np4		-	-	-	-	-	-	-	**+**	nd	**+**	**+**	**+**	-	**+**
βActin	D 9	+	+	+	+	+	-	+	+	+	+	+	+	+	+
NP3/Np4		-	-	-	-	-	-	-	**+**	-	-	**+**	**+**	**+**	**+**
βActin	D 11	+	+	+	+	+	+	+	+	+	+	+	+	dead	dead
NP3/Np4		-	-	-	-	-	-	-	-	-	-	+	-		

nd: not done

Table 2: Results of the serological tests to detect PPR antibodies in sera collected during the recombinant vaccine trial

	Dose of virus	10^5 pfu			10^3 pfu			10 pfu			0.1 pfu			control	
	n° of goat	UD32	UD33	UD41	UD34	UD35	UF1	UD36	UD37	UF2	UD38	UD39	UF3	UD40	UF5
type of test	week post vaccination														
cELISA	W 0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VNT		-	-	-	-	-	-	-	-	-	-	-	-	-	-
cELISA	W 1	-	-	-	+	+	-	-	-	-	-	-	-	-	-
VNT		-	-	-	+	-	-	-	-	-	-	-	-	-	-
cELISA	W 2	+	+	+	+	+	+	+	+	+	+	-	-	-	-
VNT		+	+	+	+	+	+	+	-	+	-	-	-	-	-
cELISA	W 3	+	+	+	+	+	+	+	-	+	+	-	-	-	-
VNT		+	+	+	+	+	+	+	-	+	-	-	-	-	-

Figure 1a

promoter direction — *Hind III* — promoter direction

tt*A**TTTA**TATT**CC**AAAAAAAAAAAAATAAAATTTCAATTTTT*aagctt*AAAAATTGAAATTTTATTTTT*

promoter direction — *Nco I*

*TTTTTTT**GG**AATA**TAAA**T*aagctcgaccatggt

Figure 1b

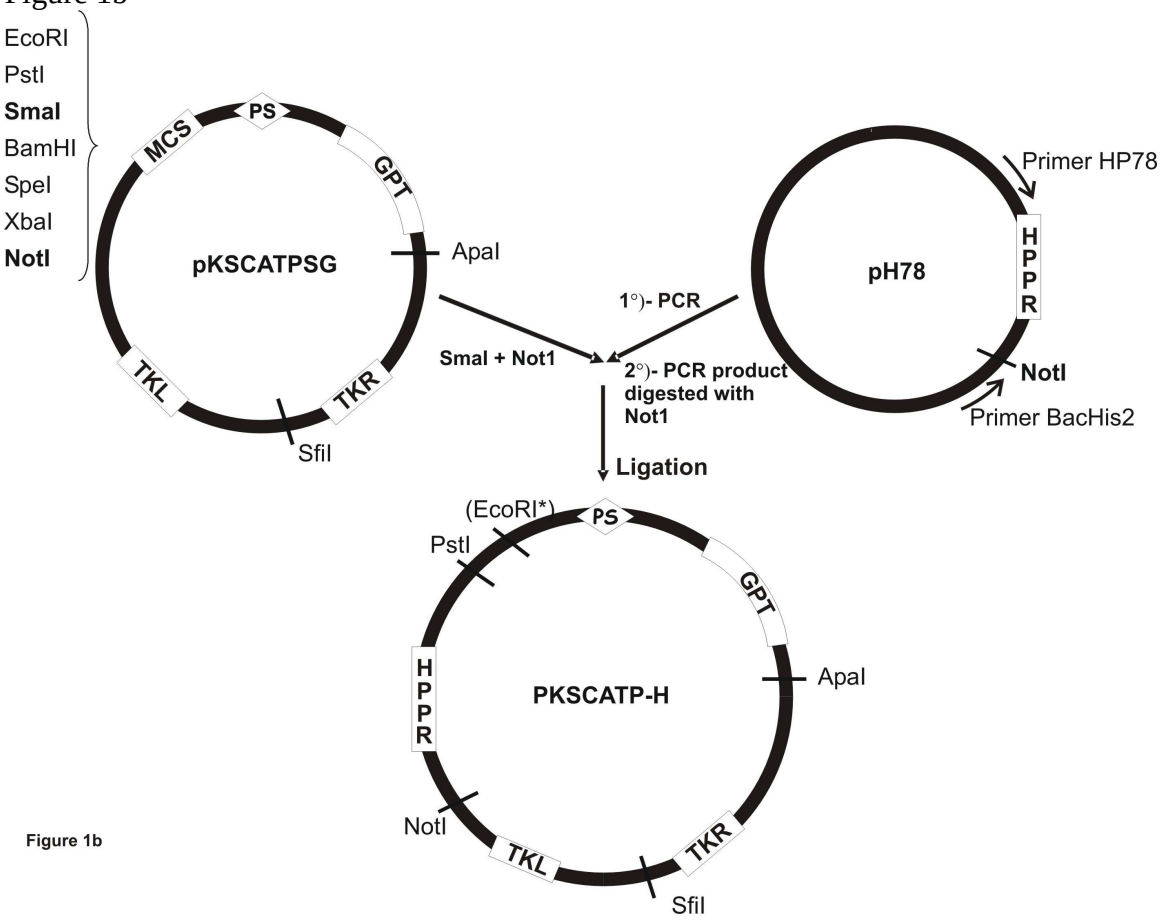


Figure 1b

Figure 2a

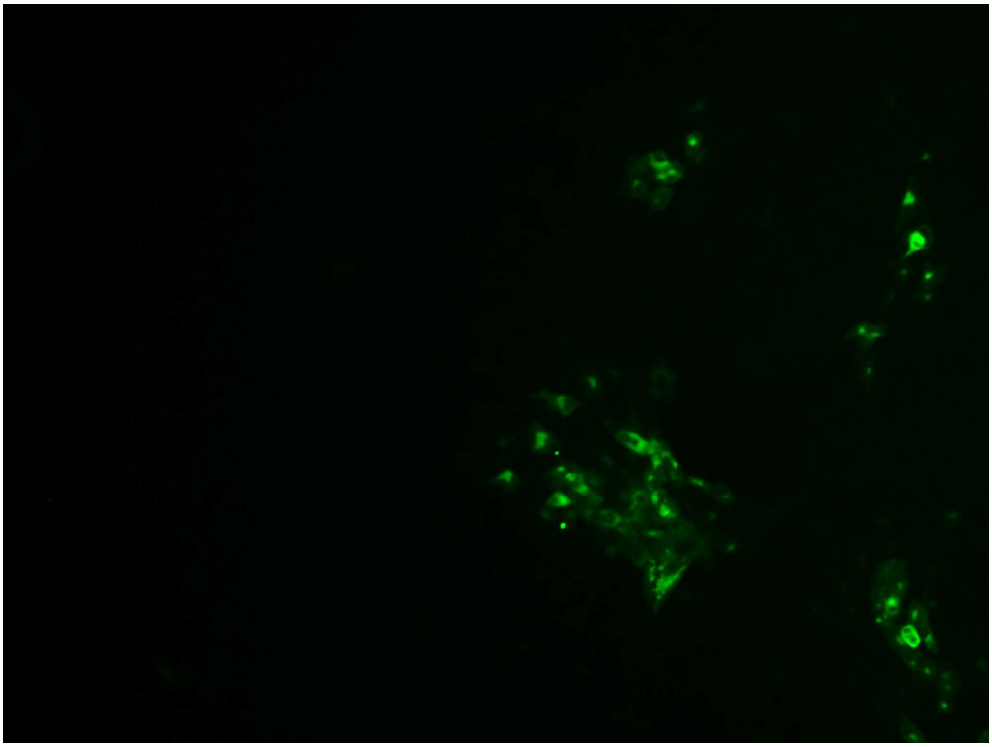
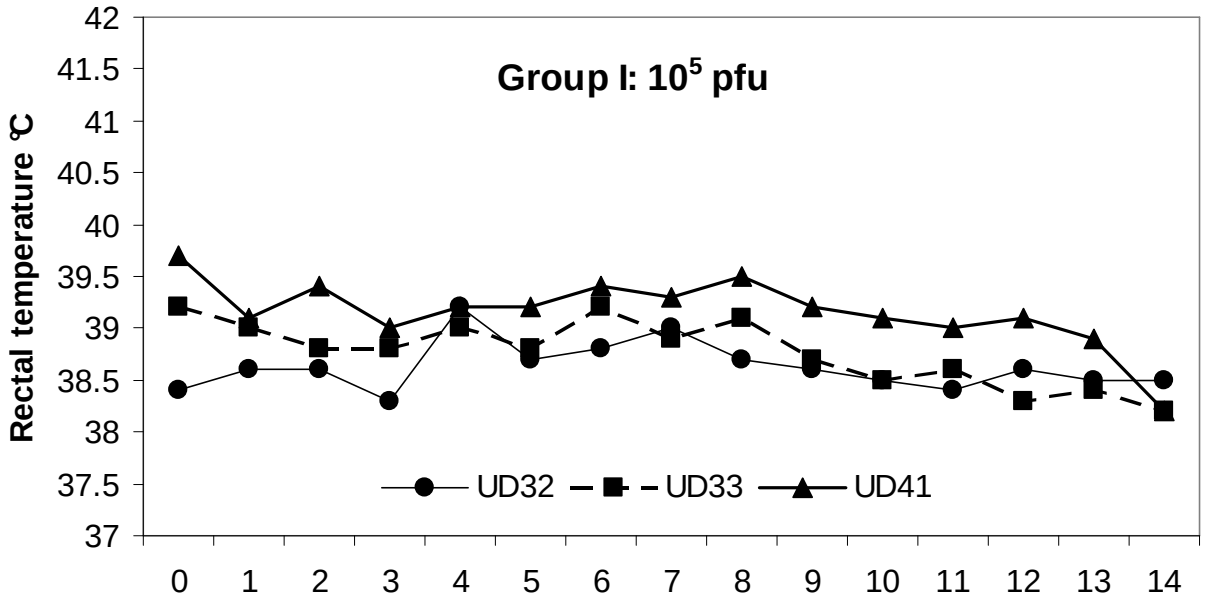


Figure 2b

Figure 3


Group I: 10^5 pfu
Rectal temperature ℃
42
41.5
41
40.5
40
39.5
39
38.5
38
37.5
37
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
UD32
UD33
UD41

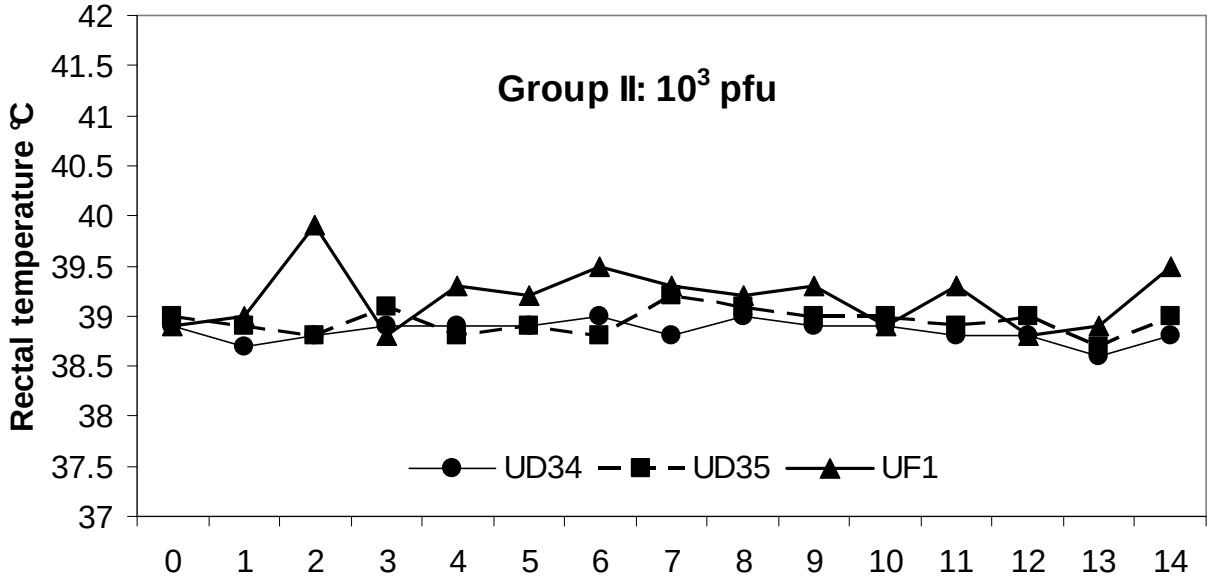
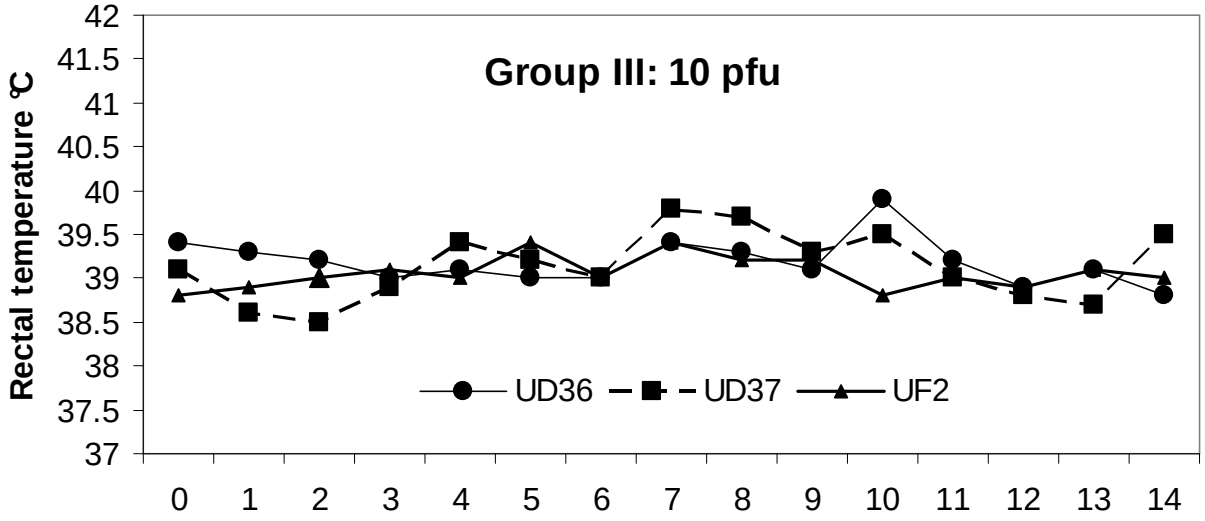

Group II: 10^3 pfu
Rectal temperature ℃
42
41.5
41
40.5
40
39.5
39
38.5
38
37.5
37
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
UD34
UD35
UF1

Figure 3


Group III: 10 pfu
Rectal temperature ℃
42
41.5
41
40.5
40
39.5
39
38.5
38
37.5
37
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
UD36
UD37
UF2

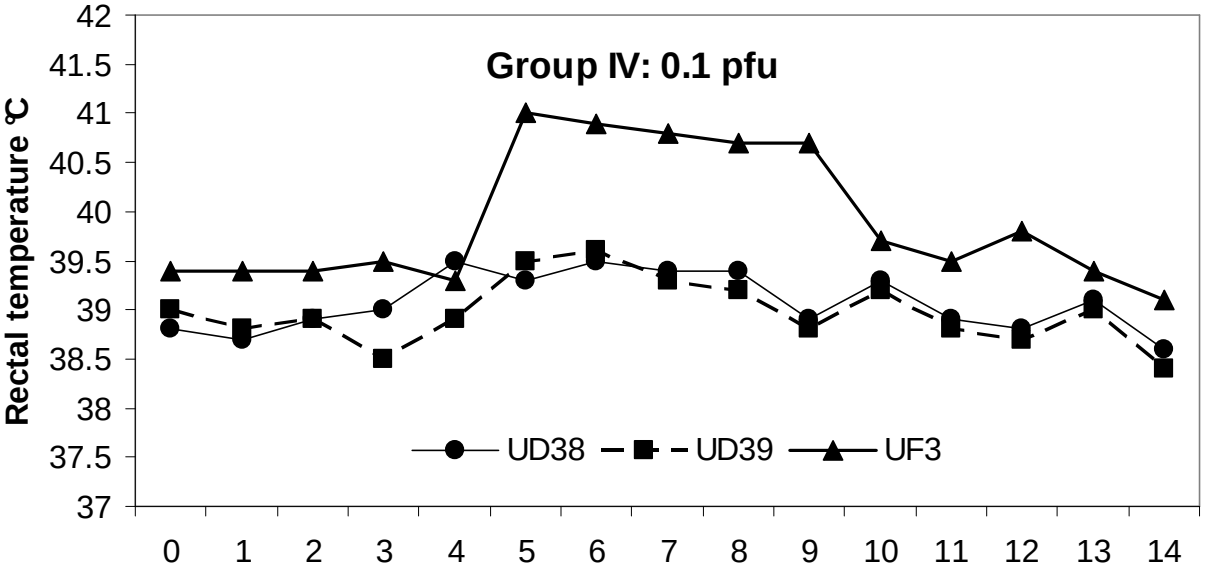
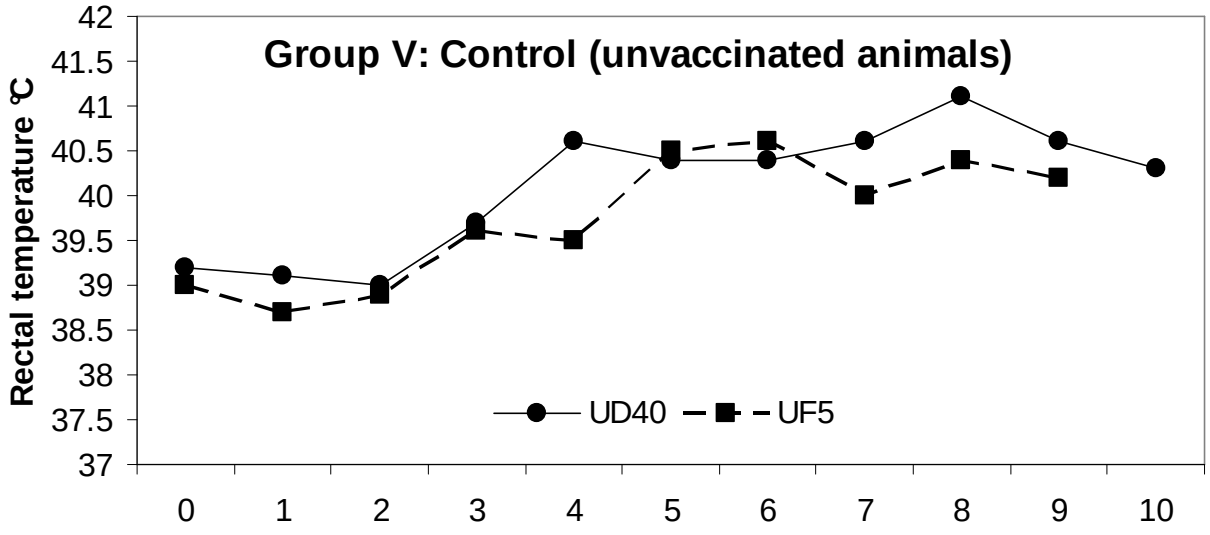

Group IV: 0.1 pfu
Rectal temperature ℃
42
41.5
41
40.5
40
39.5
39
38.5
38
37.5
37
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
UD38
UD39
UF3

Figure 3


Group V: Control (unvaccinated animals)
Rectal temperature ℃
42
41.5
41
40.5
40
39.5
39
38.5
38
37.5
37
0
1
2
3
4
5
6
7
8
9
10
UD40
UF5

Conclusion:

After vaccination with the first recombinant construction, the FPPR/capripox recombinant, no antibody anti F was detected in the serum of the vaccinated animals by the diagnostic test we have used. An anamnestic PPRV serological response was detected upon challenge. This result, in addition to the fact that the RNA of the challenge virus was detected in the white blood cells of some vaccinated animal, it also indicates that the tested recombinant virus is not providing sterile immunisation. This was the case with the recombinant capripox/H PPR using the synthetic promoter PS. As a consequence, the animals may excrete the virus so that it can propagate in the environment.

In order to increase the immunogenicity of the recombinant capripoxviruses to protect the vaccinees against the infection, we decided to work on the promoter efficacy. Promoters are short nucleic acid sequences that trigger the expression of genes. In our case, the capripox/F PPR construction was under the control of the classical vaccinia early/late p7.5 promoter while for the capripox/H PPR the early/late synthetic promoter was used as it was demonstrated to be a strong promoter (Chakrabarti *et al.,* 1997). In that case the minimum protective dose of the recombinant, although expressed in different units, was much higher than the result obtained with the first recombinant: 10 $TCID_{50}$ versus 0.1 PFU. The discrepancy of those results may be due to the quality of the protein, F being more effective than H in the protection induced by capripoxvirus, or due to the quality of the promoter. We therefore decided to test the efficiency of those two vaccinia-derived promoters along with a third one which was proved to drive synthesis of large amount of recombinant protein in the vaccinia system (Jin *et al.,* 1994).

In the next section, we describe results of the *in vitro* and *in vivo* comparison under the same conditions of three recombinant capripoxviruses containing the HPPRV gene under three different promoters.

CHAPTER THREE

Comparison of promoter efficacy

Introduction

Poxviruses are unique among DNA viruses since they complete all their replication cycle in the cell cytoplasm. Therefore the virus must bring into the cell or synthesize most of the proteins necessary for its gene expression, replication and virion assembly. Basically, the replication of poxviruses follows three phases (for review see Broyles S., 2003; Schramm and Locker, 2005). The phase one consists of the expression of early genes that help to uncoat the core and to mediate viral DNA release. The phase two consists of the replication of the viral DNA and expression of intermediate genes by transcription complexes synthesised during the early phase. The phase three concerns the expression of late genes by transcription factors expressed from the intermediate genes. The late genes produce the structural proteins of the virion. The virus coordinates its genome replication with the virion formation through the regulation of gene transcription by sequential activation of early, intermediate, early/late and late promoters. Activation sequences onto which attaches the RNA polymerase, the promoters, constitutes a key role in the control of downstream genes. Coupar *et al.* (1986) pointed out the importance of early expression of antigens by vaccinia virus for efficient priming of cytotoxic T lymphocytes (CTL) and thereby induction of long term immune response. Many foreign genes have been inserted under the control of the VV P7.5K early/late promoter either in the vaccinia virus or other poxviruses. Proteins under control of this promoter are known to stimulate both humoral and cell-mediated immune responses (Coupar *et al.*, 1986; Andrew *et al.*, 1989). Late promoters such as p11 (now referred to as gene F17) for vaccinia or the promoter for the gene of the A-Type inclusion body /ATI/ of cowpox virus direct high level protein expression (Ichihashi and Dales, 1973; Shida *et al.*, 1977). However they do not stimulate strong cell-mediated immune response.

The use of a promoter which will ensure high level of recombinant protein expression by a poxvirus at all stages of the infection will probably improve the induced immune response. With this in mind, we have compared the capabilities of three early/late promoters to direct the PPRV haemagglutin protein by the capripox virus vector: the VV p7.5, a synthetic promoter (PS) and a hybrid A–type inclusion body promoter/synthetic early promoter p7.5 (pATI16p7.5) (Chakrabarti *et al.*, 1997; Jin *et al.*, 1994)

Materials and Methods

Recombinant vaccines

Construction of the recombinant capripox/HPPR with the synthetic promoter (PS) is reported in chapter II. Here we report on the construction of the two other capripox/HPPR recombinant viruses in which the PPRV H protein gene is under the control of either the hybrid promoter pATI16p7.5 or the vaccinia promoter p7.5. The transfer vector plasmid pKSCATP-H was used to construct the recombinant capripox/HPPR with the synthetic promoter PS (see figure 1a and 1b of manuscript 3). In this plasmid, both PPRV H and the selection marker GPT genes are under the control of PS, a double early/late synthetic promoter. This plasmid was used in the construction of the two other capripox/HPPR recombinants in which the promoter element controlling the PPRV H gene is replaced by either the hybrid promoter pATI16p7.5 or the vaccinia promoter p7.5. It has been digested by PstI (Biolabs), then repaired with T4 DNA Polymerase (Biolabs) and digested again by HindIII (Biolabs). This double digestion eliminates the fragment of the promoter contolling H PPR gene (see figure 1a and 1b of manuscript 3). The plasmid pBSFJ2-16 (gift from Dr H. Shida, Kyoto University, Japan) contains the hybrid promoter pATI16p7.5. It was mutated to insert a HindIII restriction site upstream the pATI sequence. The new plasmid, pBSF2-16Δ, was digested by EcoRI, repaired with T4 DNA polymerase and digested by HindIII to release the hybrid promoter fragment. It was gel purified along with the double digested plasmid pKSCATP-H using the Qiaquick gel extraction kit (Qiagen). Vector and insert were ligated with the DNA ligation kit (Amersham). DH5a E.coli strain was transformed with the ligation product. Colonies were directly screened by PCR with HP47 and Ati176 primers to produce a fragment of about 1500 bp. Positive clones were grown overnight at 37°C, 250 rpm on LB medium containing 1% ampicilline DNA was extracted from culture with Wizard Plus SV Minipreps DNA purification system (Promega). Then the clone was sequenced on ABI Prism 377 to confirm the construction. A large amount of the plasmid DNA was produced and extracted with Endofree maxi prep (Qiagen). The new plasmid is named pCA216-H. Another transfer vector plasmid was constructed by ligating into the HindIII/ECoRI doubled digested pKSCATP-H the promoter p7.5 that was released from the plasmid JC-35 (see in article 1). The resultant plasmid obtained was named pHPPRp7.5. Using the method described in Article 1 and manuscript 3, both pCA216-H and pHPPRp7.5 were used

to generate new capripox/H-PPR recombinant viruses which express the PPRV H protein under the control of the hybrid promoter pATI16-P7.5 or p7.5.

In vitro evaluation of H expression by the different recombinant capripox/HPPR viruses

Culture cell flasks of 75 cm², containing OA3Ts cell monolayer at 80% of confluence were inoculated with each of the recombinant capripox/HPPR virus at $10^{4.5}$ $TCID_{50}$. The parental capripox virus KS1 was used as the negative control of the experiment. After 5 days of incubation at +37°C and 5% CO_2, the cells were trypsinised and washed in PBS-5% horse serum-0.005% sodium azide-0.1% saponine (incubation buffer). The cells were then incubated for 30 minutes at +4°C in presence of one of the following antibodies appropriately diluted in the incubation buffer:

- mouse monoclonal antibody anti-HPPR (IAH, Pirbright) at 1/100
- sheep anti-pox serum (CIRAD) at 1/200
- mouse negative serum at 1/400
- sheep negative serum at 1/200

The stained cells were then washed twice with the incubation buffer. They were incubated again for 30 minutes at +4°C with conjugate either anti-sheep FITC (ref F0135, Dako) diluted at 1/40 or anti-mouse TC (ref M32006, Caltag) diluted at 1/100 in the incubation buffer. After two washes in the same buffer, the cells were finally re-suspended in FACs flow buffer and analysed in a FACsorter (Becton Dickinson).

Evaluation of the different capripox/HPPR recombinant viruses for protection of Goats against PPR

Eighty (80) goats of both sexes, aged between 8-16 months, were purchased from the high land part of Ethiopia, expected to be “free” of PPRV infection. They were brought to the National Veterinary Institute of Ethiopia (NVI) in Debre Zeit. They were bled for serum collection. All samples were analysed at CIRAD-EMVT in Montpellier and NVI for the

detection of antibodies to the hemagglutinin protein of PPRV and RPV using specific PPRV and RPV monoclonal antibody-based competitive enzyme-linked immunosorbent assays (cELISA) developed by Anderson *et al.* (1991). The animals were treated by broad-spectrum anthelmentics (5.00 mg Albendazole per kg bodyweight). Following the screening test, those sera which were free from PPR or RPV antibodies were selected, tagged and randomly allocated to five different groups. They were immunised with different vaccines at doses varying from 0.1 to 10^3 $TCID_{50}$/animal by subcutaneous route (see table 1). The capripox/F PPR recombinant and the classical attenuated PPR vaccine were included as positive control in the experiment. Housing of animals was a well-ventilated and rodent proof. Three weeks after vaccination, animals were challenged with the virulent PPRV India 94/1 Vero 3. They were submitted to daily clinical observation. They were bled at day zero, 15, 30 and 45, last day of the clinic observation.

group / **dose**	**Rec-H-p7.5 (G1)**	**Rec-H-PS (G2)**	**Rec-H-pATI16p7.5 (G3)**	**Capripox-FPPR (G4)**	**Classical attenuated PPR vaccine. (G5)**
10^3	3	3	3	0	3
10^2	3	3	3	0	0
10	3	3	3	0	0
1	3	3	3	0	0
0.1	3	3	3	3	0
0 (negative controls)	2	2	2	2	2
Total	**17**	**17**	**17**	**5**	**5**

Table 1: Experimental design for the comparison of promoters

Result and discussion

In vitro quantitative analysis of promoter efficacy

The comparative analysis of H-PPRV expression in OA3 TS cells infected with the three recombinant capripoxviruses using different promoters is shown on figure 1. The fluorescence obtained with KS1 represents the background. The highest level of H production was observed with the recombinant where the gene of this protein is under the control of the promoter p7.5, followed by that with promoter PS and pATI16p7.5.

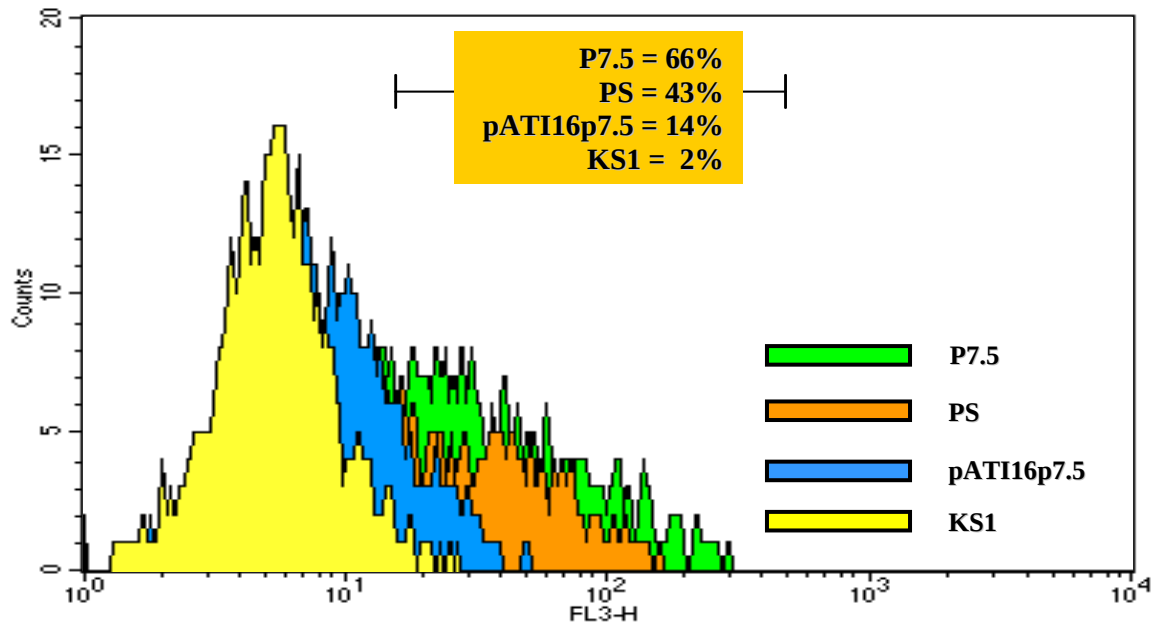


Figure 1: *In vitro* comparison of expression level of PPRV H protein under different promoters in capripox genome. Vero cells were infected at the same MOI with different recombinant capripoxviruses in comparison with non recombinant capripoxvirus (KS1). The H gene was under p7.5, PS or pATI16p7.5 promoters.

Screening test for animal selection

All sera samples collected from 80 (eighty) goats were tested for the presence of antibodies to the haemagglutinin protein of PPRV and RPV. The results showed that seven out of eighty (8.7%) were positive for PPRV antibodies and four other (5%) were doubtful (see

table 2). All positive and doubtful animals were immediately culled from the group. The remaining 69 (sixty nine) were negative for PPRV. All 80 (eighty) goats were negative for RPV (table 2). Those sixty nine (69) PPR seronegative goats were selected for the animal experiment.

No. of Samples	cELISA Antibody test	Number Positive	Percentage Positive	Doubtful	Percentage Doubtful	Total Negative
80	PPRV	7	8.7	4	5	69
80	RPV	0	0	0	0	80

Table 2: Peste des Petits Ruminants Virus (PPRV) and Rinderpest Virus (RPV) antibodies in the sera collected from test animals, Debre-Zeit, Ethiopia.

Evaluation of the Different Capripox/HPPR Recombinant viruses in the protection of Goats against PPR

Vaccinated animals remained healthy after vaccination. Three weeks later, they were submitted to PPR virulent challenge along with their control contacts. Three to four days later, all controls became sick with the following signs summarized in table 3: hyperthermia (rectal temperature at above 39°C), nasal discharge, lacrymation, mouth ulcerations and coughing. Some showed diarrhoea. There were important changes on the lymph nodes, showing different level in increase of size (table 3). Three out of 10 of those control goats died before the end of the experiment. All animals which were vaccinated with either the classical attenuated PPR vaccine or the recombinant capripox/F PPR recombinant virus were perfectly protected against the virulent challenge. Animals which have been inoculated with the recombinant in which the promoter p7.5 is used resisted to the challenge except the one which had received the dose of 0.1 $TCID_{50}$. This later had rectal temperature greater than 39 °C for 3 days (see figure 2). Among the goats which were inoculated with recombinant virus which has the synthetic promoter, those with doses of 0.1 and 1 $TCID_{50}$ virus had fever for a couple of days. The group of animals which had received the recombinant with the hybrid promoter was the most reactive group after the virulent challenge. Indeed, apart from animals which were inoculated with the recombinant at

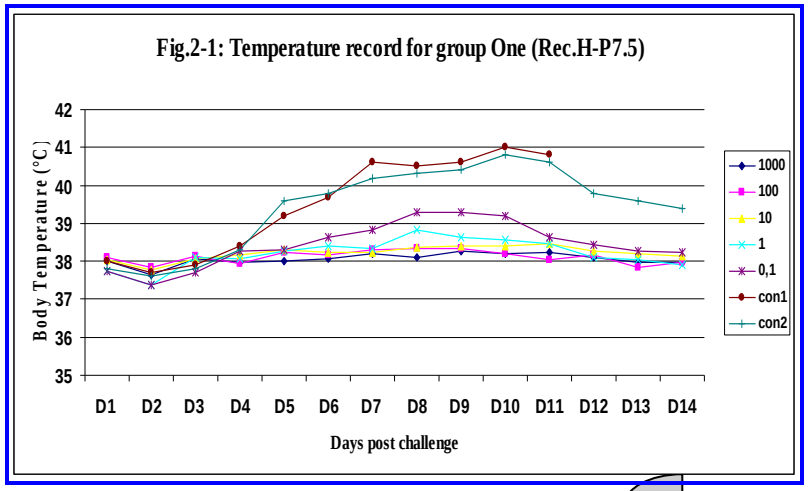
the dose of 1000 $TCID_{50}$, all other had fever for at least one day. One of the animals with the dose of 0.1 $TCID_{50}$ was so sick that it was euthanized humanely.

Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Mean T° record	37.9	38.6	39.1	39.7	40.2	40.4	40.2	40.0	39.8	39.7	39.2	39.0	39.3	39.1
Nasal discharge				6/10	6/10	6/10	5/10	3/10						
Lacrimation					6/10	6/10	5/10							
Mouth ulcerations					5/10	5/10	5/10	4/10						
Coughing				3/10	5/10	5/10	4/10	3/10	3/10					
Diarrhoea								3/10	3/10	2/10	1/10			
Lymph nodes					**Swollen**									
Mortality*										**1**	**1**	**1**	**2**	

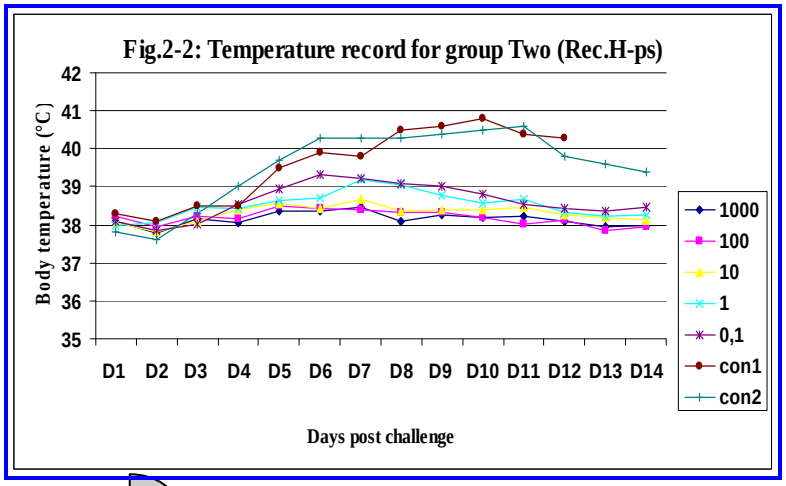
Table 3: Average body temperature records, necropsy findings and mortality of unvaccinated control animals from all experimental groups (2 control goats in each group). * Number of animals found dead or sacrificed for human reason.

Figure 2: Temperature records of the five goat groups vaccinated with recombinant capripoxviruses. Three weeks after capripoxvirus vaccination, the goats were challenged with a virulent strain of PPRV. Curves represented the average result for the corresponding group.

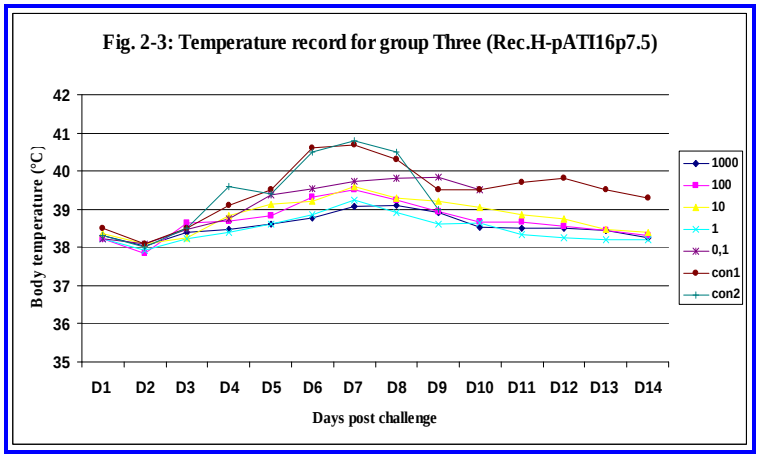
A. Comparison of groups one, two and three.



a) Rec H-P7.5

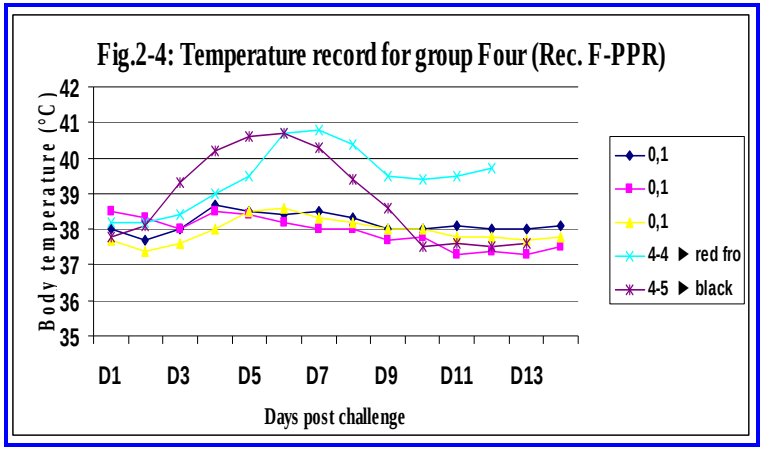


b) Rec H-PS

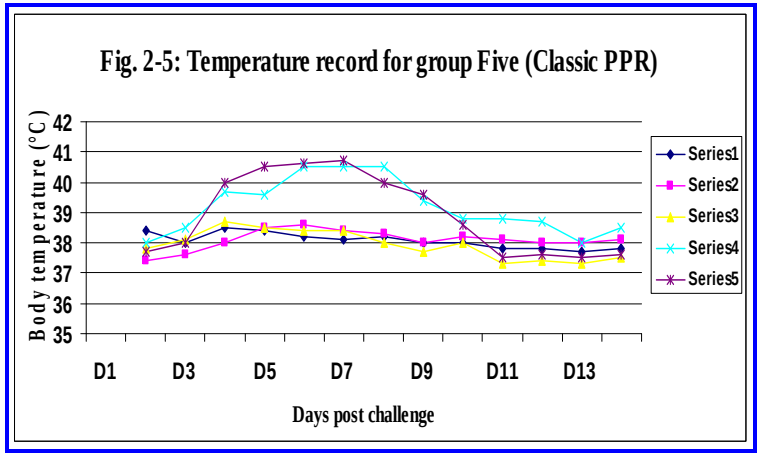


c) Rec.H- pATI16p7.5

B. Comparison of groups four and five



a) Rec F-PPR



b) Classic PPR

Conclusion

In an attempt to develop a very efficient recombinant capripox-based vaccine expressing H of PPRV, we have compared the relative strength of three poxvirus promoters to drive the production of this recombinant protein. It is well known that in the vaccinia vector system both the amount of the antigen and the timing of its synthesis impact on the quality of the induced immunity, cytotoxic T lymphocytes being primed mainly if the antigen is synthesized at early stages of the virus infection (Coupar *et al.,* 1986). This may be true for all poxviruses. Therefore, in our experiment with capripox virus, we have compared three poxvirus promoters which are effective at both early and late stages of virus infection:

- (a) the vaccinia p7.5 promoter which is widely used in the construction of poxvirus-based recombinant vaccines.
- (b) a compact synthetic promoter which was proved to be stronger, about 50 times, than p7.5 in driving expression of protein (Chakrabarti *et al.,* 1997). Antigens produced under the control of this promoter induce in animals both humoral and cellular immune responses as it is the case for the p7.5 promoter (Sutter *et al.,* 1994).
- (c) a hybrid promoter (pATI16p7.5) made with the strong cowpox late promoter ATI and the early promoter sequence of p7.5 tandemly repeated 16 times. This promoter was shown to support high level of recombinant protein production (Jin *et al.,* 1994).

The production of the PPRV H protein by the three recombinant capripox/ H PPR was assessed in cell culture with the FACsorter after staining the cells with fluorescent dye-labeled antibody. This study has shown that for the PPRV H production in the capripox virus system, p7.5 promoter was the most efficient. It was followed by the synthetic promoter. In this system, pATI16p7.5 was the weakest promoter. The three different recombinant capripox/H PPR viruses were tested in the Debre-Berhan goat breed in Ethiopia. Upon challenge with the virulent strain PPRV India 1994/1, the minimum effective dose was 1 $TCID_{50}$ for the recombinant with the promoter p7.5, 10 $TCID_{50}$ for the recombinant with the synthetic promoter and 1000 $TCID_{50}$ for the recombinant with pATI16p7.5. These results correlate well with the *in vitro* quantitative analysis of the promoter efficacy. However, they do no confirm previous data that were found with the synthetic and the hybrid promoters in the vaccinia system where they were directing high level of gene expression (Jin *et al.,* 1994; Chakrabarti

et al., 1997). The minimal effective dose of the recombinant capripox/H PPR with the synthetic promoter, 10 $TCID_{50}$, which was found in the Ethiopian experiment, confirmed the result of the previous test carried out on goats in the UK and reported in manuscript 3. In the animal experiment to compare the different capripox/H PPR recombinants, was included also the capripox/F PPR recombinant as a positive vaccine control with the classical attenuated PPR vaccine. Goats which were immunised with those vaccines were protected. The recombinant capripox/F PPR virus was used at the dose of 0.1 $TCID_{50}$/goat. These results reproduced the ones obtained in experimental trials carried out in Article 1 in which a dose of 0.1 PFU/goat was found protective. In both experiments, the PPRV F gene was under the control of the promoter p7.5. The minimal effective dose of the similar construction with the PPRV H gene to fully protect goats against a virulent PPR challenge was 1 to 10 $TCID_{50}$. This confirms that fusion protein of morbilliviruses expressed in a context of capripoxvirus is more potent than H in the induction of protective immune response in the hosts.

GENERAL DISCUSSION

AND CONCLUSION

Peste des petits ruminants (PPR) is a highly viral contagious disease which is threatening the production of nearly a billion of sheep and goats in Africa, Asia, the Middle and Near East. It is one of the diseases targeted by the FAO in its EMPRESS programme which aims at controlling some important transboundary animal diseases. Its economic importance has been highlighted by an international study which has identified it as one of the priority animal diseases to be considered in poverty alleviation policy in areas where it is endemic (Perry *et al.,* 2002). This study has considered small ruminants as the only hosts of PPRV. However, considering the fact that this virus can infect and cause disease in cattle, buffaloes and camels in some unknown circumstances PPR has therefore even higher priority, particularly in the current situation where vaccination against rinderpest in cattle has been stopped (Mornet *et al.;* 1956, Govindarajan *et al.,* 1997; Roger *et al.,* 2000; 2001). For most of the countries where it is endemic, the disease control measure easy to be implemented is the vaccination. There is an effective attenuated PPR vaccine which provides a life long immunity to inoculated animals (Diallo *et al.,* 1987). Unfortunately, and as pointed out in a recent report, very few countries are implementing systematic vaccination campaigns to protect sheep and goats against PPR (Diallo, 2004). This may be explained by many constraints of which are: lack of information and the cost of the logistic for small ruminant vaccination. Indeed, the majority of small ruminants are owned by poor farmers. They have little contact with field veterinarians and also cannot afford vaccination costs if they are not born by governments. Although sheep and goats are important for the livelihoods of those owners, unfortunately on international trade scale they are not considered as valuable individually as for cattle. Moreover, nearly all small ruminant trade are carried out between countries within PPR endemic zones so this disease, although being in the list of important animal diseases to be notified to the OIE, does not constitute a strong constraint in international animal exchange as do rinderpest and FMD for example. All those perceptions on small ruminants and PPR make unlikely the implementation of an internationally-funded global eradication programme in line to the strategy which has almost been completed for rinderpest. In addition, considering that small ruminant populations are very scattered and that a high number of animals are concerned by the vaccination against PPR, there is a need of an important logistic for vaccination campaigns that becomes costly. A way to cut down this cost is to address the control of more than one important disease in a single operation. The objective of our work was to develop such a tool by constructing a recombinant capripox-based PPR vaccine able to protect small ruminants against both PPR and capripox, another

transboundary animal disease which is also part of the group of animal diseases to be notified to the OIE as PPR.

In a first step, we have inserted into the capripoxvirus genome the cDNA corresponding to the fusion protein of PPRV. The resultant recombinant virus that was obtained was tested very effective as a dual vaccine to protect goats against both PPR and capripox infections. We then produced three other capripox-based recombinant viruses expressing the PPRV haemagglutinin protein under the control of three different poxvirus early/late promoters. Assuming that those new recombinants will not differ from the capripox/F PPR in their capacity to protect small ruminants against capripox infection, the foreign gene insertion site being the same in the TK gene of the capripox virus for all, they were tested only for their capacity to protect against PPR. Among the three recombinant capripox/H PPR viruses, the one where the expression of H was under the control of the promoter p7.5 was the most potent since its minimum effective dose was estimated to be 1 $TCID_{50}$/animal, ten times more than the capripox/F PPR in which the same promoter was used. Although no quantification of the F production was made and compared with that of H, the difference in the efficacy of the capripox/F PPR and capripox/H PPR recombinant viruses using the same promoter may be due to the more immunoprotective capacity of F than that of H in the context of capripoxvirus expression. With the objective to improve the quality of the recombinant capripox/H PPR, the efficiency of three promoters in driving the expression of H was compared. In addition to the classical vaccinia virus promoter p7.5, the two other were the synthetic early/late promoter (PS) and the hybrid promoter pATI16p7.5. Previous reports indicated that the last two promoters were by far most efficient in the expression of foreign proteins by the vaccinia virus (Chakrabarti *et al.*, 1997; Jin *et al.*, 1994). We expected that one these two promoters will direct high level expression of PPRV H by the capripoxvirus, expectation based on the assumption that a promoter of a poxvirus is functional with all other poxviruses (Tripathy and Wittek, 1990; Kumar and Boyle, 1990). Unfortunately, our result was the opposite of what was obtained with the vaccinia virus system: the p7.5 was the most efficient. Although we have not tested the expression of the PPRV H in the vaccinia virus system, we think that the non-expected result we have obtained is linked to the very low efficiency of PS and pATI16p7.5 in capripox virus. Indeed although it is claimed that transcription factors and promoter function are conserved between poxviruses, at least within a subfamily (Broyles, 2003), subtle differences in the efficiency of gene expression processes may exist between viruses. A comparative study has demonstrated differences between fowlpox and vaccinia

viruses in the efficiency of promoters to drive expression of the same gene (Boyle, 1992). Following our results, research has been launched in CIRAD-EMVT to look for a capripoxvirus promoter that may be used for efficient foreign gene expression by capripox-based recombinant viruses.

Meantime, the recombinants we have developed will be better evaluated during the project which has just started. One of the objectives of this project is to determine the duration of immunity provided by both capripox/F PPR and capripox/ H PPR recombinant viruses. They will have a real interest if they prove to confer a long-term immunity, at least comparable to the one induced by the classical PPR vaccine, i.e. about 3 years. Since these recombinant vaccines are to be used in PPR and capripox endemic areas, some animals may have either PPR or capripox pre-existing antibodies. The effect of this pre-existing immunity on the efficacy of capripox-based PPR recombinant vaccine has to be studied. In the case of vaccinia-based recombinant, there are conflicting reports on the effect of pre-existing antibodies. Some reports indicated that the recombinant vaccine is still effective in the presence of antibodies directed against either the foreign gene or the vaccinia vector itself (Flexner *et al.*, 1988; Ramírez *et al.,* 2000). However, others indicated that strong immune response against viral vector proteins was associated with a lower effective immune response against vector-expressed foreign antigens (Rooney *et al.,* 1988; Kundig *et al.,* 1993).

One of the possible features of the capripox-based PPR recombinant vaccine is the thermotolerance since the areas for its potential use are of hot climate. A study has to be carried on both liquid and freeze-dried product to determine the conditions of storage and use of the recombinant capripox-PPR vaccine in the field.

A major disadvantage of the current classical attenuated PPR homologous vaccine is that the antibody response it induces cannot be distinguished from that following a natural infection. This makes the sero-epidemiosurveillance of the disease impossible in endemic areas where a vaccination programme has to be or is being implemented. A way to combine both activities, vaccination and serosurveillance, for the better management of the disease would be the use of DIVA vaccines, the acronym used for vaccines which enable **d**ifferentiation between **i**nfected and **v**accinated **a**nimals (van Oirschot JT., 1999). The capripox-based PPR recombinant vaccine which carries only F and H protein genes, in conjunction use with a PPR ELISA kit in which the antigen is the PPRV nucleoprotein (Libeau *et al.,* 1995), constitutes a DIVA vaccine which will facilitate the control of PPR by allowing both vaccination and seroepidemiosurveillance activities at the same time.

REFERENCES

Abimiku, A.G., Franchini, G., Tartaglia, J., Aldrich, K., Myagkikh, M., Markham, P.D., Chong, P., Klein, M., Kieny, M.-P., Paoletti, E., Gallo, R.C. and Robert-Guroff, M., (1995). HIV-1 recombinant poxvirus vaccine induces cross-protection against HIV-2 challenge in rhesus macaques. *Nature Medicine* **1**, 321–329.

Ali, A.A., Esmat, M., Attia, H., Selim, A. and Abdel-Hamid, Y.M., (1990). Clinical and pathological studies on Lumpy Skin Disease in Egypt. *The Veterinary Record*, **127**, 549-550.

Alkahatib, G., Massie, B. and Briedis, D.J., (1988). Expression of bicistronic measles virus P/C mRNA by using hybrid adenoviruses: levels of C protein synthesised *in vivo* are unaffected by the presence or absence aof the upstream P initiator codon. *Journal of Virology* **62**, 4059–69.

Amjad, H., Qamar-ul-Islam, Foryth, M., Barrett, T., and Rossiter, P.B., (1996). Pets des Petits Ruminants in goats in Pakistan. *The Veterinary Record* **139**(5), 118-9

Andersson, S., Makitalo, B., Thorstensson, R., Franchini, G., Tartaglia, J., Paoletti, E., Putkonen, P. and Biberfeld, G. I., (1996). Immunogenicity and protective efficacy of a human immunodeficiency virus type 2 recombinant canarypox (ALVAC) vaccine candidate in cynomolgus monkeys. *Journal of Infectious Diseases* **174**(5), 977-85.

Andrew, M.E., Coupar,B.E.H. and Boyle, D.B. (1989). Humeral and cell mediated immune response to recombinant vaccinia viruses in mice. *Immunology and Cell Biology* **67,** 331-337

Aspden, K., van Dijk, A.A., Bingham, J., Cox, D., Passmore, J-A and Williamson, A-L., (2002). Immunogenicity of a recombinant lumpy skin disease virus (Neethling vaccine strain) expressing the rabies virus glycoprotein in cattle. *Vaccine* **20**, 2693-2701.

Aspden, K., Passmore, J-A., Tiedt, F. and Williamson, A-L., (2003). Evaluation of lumpy skin disease virus, a capripoxvirus, as a replication-deficient vaccine vector. *Journal of General Virology* **84,** 1985-1996

Bailey, D., Banyard, A., Dash, P., Ozkul, A. and Barrett, T., (2005). Full genome sequence of Peste des Petits Ruminants virus, a member of the Morbillivirus genus. *Virus Research* **110**, 119–24.

Banerjee, A.K., (1987). Transcription and replication of rhabdoviruses. *Microbiology Review* **51**, 66–87.

Baron, M.D. and Barrett, T. (1995). Sequencing and analysis of the nucleocapsid (N) and the polymerase genes and the terminal extragenic domains of the vaccine strain of rinderpest virus. *J. Gen. Virol.,* **76**: 593-602.

Baron, M.D. and Barrett, T., (2000). Rinderpest virus lacking the C and V proteins show specific defects in growth and transcription of viral RNAs. *Journal of Virology* **74**, 2603–11.

Baron, M.D., Shaila, M.S., Barrett, T., (1993). Cloning and sequence analysis of the phosphoprotein gene of rinderpest virus. *J. Gen. Virol.,* **74**, 299-/304.

Barrett, T., Shrimpton, S.B., Russell, S.E.H., (1985). Nucleotide sequence of the entire protein coding region of Canine Distemper Virus polymerase-associated (P) protein messenger RNA. *Virus Res.,* 3: 367-/372.

Barrett, T., Banyard, AC. and Diallo, A., (2006). Molecular biology of the morbilliviruses. In: Rinderpest and Peste des Petits Ruminants, Virus Plagues of large and Small Ruminants. T. Barrett, P.P. Pastoret and W.P. Taylor (eds.), Academic Press, Elsevier, 31-67

Bellini, W.J., Englund, G., Rozenblatt, S., Arnheiter, H. and Richardson, C.D., (1985). Measles virus P gene codes for two proteins. *Journal of Virology* **53**, 908–19.

Bhanuprakash, V., Indrani, B.K., Hedge, R., Kumar, M.M. and Moorthy, A.R. (2004). A classical live attenuated vaccine for sheep pox. *Tropical Animal Health and Production* **36** (4), 307-320

Binns, M. and Smith, G.L., (1992). Recombinant poxviruses, *CRC Press, Boca Raton* (1992).

Black, D.N., Hammond, J.M. and Kitching, R.P. (1986). Genomic relationship between Capripoxviruses. *Virus Research* **5**, 277-292.

Blain, F., Liston, P. and Briedis, D.J., (1995). The carboxy-terminal 18 amino acids of the measles virus hemagglutinin are essential for its biological function. *Biochemical Biophysical es. Commun* **214**, 1232–8.

Blanchard, T.J., Alcami, A., Andrea, P. and Smith, G.L. (1998). Modified vaccinia virus Ankara undergoes limited replication in human cells and lacks several immunomodulatory proteins: implications for use as a human vaccine. *Journal of General Virology* **79 (5),** 1159-1167.

Blau, D.M. and Compans, R.W., (1995). Entry and release of measles virus are polarized in epithelial cells. *Virology* **210**, 91–9.

Blumberg, B. and Kolakofsky, D., (1981). Intracellular vesicular stomatitis virus leader RNAs are found in nucleocapsid structures. *Journal of Virology* **40**, 568–76.

Bolt, G., Alexandersen, S. and Blixenkrone-Møller, M., (1995). The phosphoprotein gene of a dolphin morbillivirus isolate exhibits genomic variation at the editing site. *Journal of General Virology* **76**, 3051–8.

Bolt, G., (2001). The measles virus (MV) glycoproteins interact with cellular chaperones in the endoplasmic reticulum and MV infection upregulates Chaperone expression. *Archives of Virology* **146**(11), 2055-68

Bourdin, P., (1979). Problèmes posé par la pathologie virale du mouton en zone sahélienne et soudano-sahélienne. *Revue D'Elevage et de Médecine Vétérinaire des Pays Tropicaux*, **32**(2) : 123-9

Bourdin, P et Laurent-Vautier, A., (1967). Note sur la structure du virus de la peste des petits ruminants. *Rev. Elev. Méd. Vét. Pays Trop.*, **20**: 383-386.

Boyle, DB., (1992) Quantitative assessment of poxvirus promoters in fowlpox and vaccinia virus recombinants. *Virus Genes.* **6**(3):281-90.

Boyle, D.B., Anderson, M-A., R., Voysey, R.and Coupar, B.E.H., (2004). Construction of recombinant fowlpox viruses carrying multiple vaccine antigens and immunomodulatory molecules. *Bio Techniques*, **37**(1), 104-111.

Brochier, B. Costy, F. Pastoret, PP., (1995). Elimination of fox rabies from Belgium using a recombinant vaccinia-rabies vaccine: an update. *Vet Microbiol.*, **46**:269-79.

Brochier, B., Aubert, MF., Pastoret, PP., Masson, E., Schon, J., Lombard, M., Chappuis, G., Languet, B., Desmettre, P., (1996). Field use of a vaccinia-rabies recombinant vaccine for the control of sylvatic rabies in Europe and North America. *Rev Sci Tech.*, **15**:947-70.

Brown, DD., Rima, BK., Allen, IV., Baron, MD., Banyard, AC., Barrett, T., Duprex, WP., (2005a). Rational attenuation of a morbillivirus by modulating the activity of the RNA-dependent RNA polymerase. *J Virol*., **79**:14330-14338

Brown, D.D., Collins, F.M., Duprex, W.P., Baron, M.D., Barrett, T. and Rima, B.K., (2005b). 'Rescue' of mini-genomic constructs and viruses by combinations of morbillivirus N, P and L proteins. *Journal of General Virology* **86**, 1077–81.

Broyles, S.S., (2003). Vaccinia virus transcription. *Journal of General Virology*, **84**: 2293-2303

Cadoz, M., Strady, A., Meignier, B., Taylor, J., Tartaglia, J., Paoletti, E. and Plotkin, S., (1992). Immunisation with canarypox virus expressing rabies glycoprotein. *Lancet.* **339**, 1429–1432.

Calain, P. and Roux, L., (1993). The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. *J. Virol.*, **67**: 4822-4830.

Carroll, M.W. and Moss, B., (1997). Poxviruses as expression vectors, *Current Opinion In Biotechnology* **8**, 573–577.

Carn, V. M., (1993). Control of Capripox infections. *Vaccine* **11**(13), 1275-1279.

Carroll, M. W., Overwijk, W., Surman, D., Tsung, K., Moss, B. and Restifo, M., (1998). Construction and characterization of a triplerecombinant vaccinia virus encoding B7-1, IL-12, and a model tumour antigen. *Journal of National Cancer Institute* **90**, 1881–1887.

Cartee, T.L., Megaw, A.G., Oomens, A.G. and Wertz, G.W., (2003). Identification of a single amino acid change in the human respiratory syncytial virus L protein that affects transcriptional termination. *Journal of Virology* **77**, 7352–60.

Chakrabarti, S., Sisler, JR. and Moss, B., (1997). Compact, synthetic, vaccinia virus early/late promoter for protein expression. *Biotechniques*, 23:1094-7

Chip, S., (1993). An economic analysis of the prevention of Peste des Petits Ruminants in Nigerien goats. *Preventive veterinary medicine* **16** (2), 141-150

Choppin, P.W. and Scheid, A., (1980). The role of viral glycoproteins in adsorption, penetration, and pathogenicity of viruses. *Rev. Infect. Dis.*, **2**: 40-61

Coronel, E.C., Murti K. G., Takimoto T., and Portner A., (1999). Human parainfluenza virus type 1 matrix and nucleoprotein genes transiently expressed in mammalian cells induce the release of virus-like particles containing nucleocapsid-like structures. *J. Virol*. **73:**7035-7038

Coronel, E.C., Takimoto, T., Murti, K.G., Varich, N. and Portner, A., (2001). Nucleocapsid incorporation into parainfluenza virus is regulated by specific interaction with matrix protein. *Journal of Virology* **75**, 1117–23.

Coupar, B.E.H.; Andrew, M.E.; Both, G.W. and Boyle, D.B., (1986). Temporal regulation of hemagglutinin expression in vaccinia virus recombinants and effects on the immune response. *European Journal of Immunology* **16**, 1479-1487.

Cox, W.I., Tartaglia, J. and Paoletti, E., (1993). Induction of cytotoxic T lymphocytes by recombinant canarypox (ALVAC) and attenuated vaccinia (NYVAC) viruses expressing the HIV-1 envelope glycoprotein. *Virology* **195**, 845–850.

Crowley, J., Dowling, P., Mennoma, J., Silvermann, J., Schuback, D., Cook, S., and Blumberg, B., (1988). Sequence variability and function of measles virus 3' and 5' ends and intercistronic regions. *Virology,* **164**: 498-506.

Curran, M.D., Rima, B.K., (1992). The genes encoding the phosphoprotein and matrix proteins of phocine distemper virus. *J. Gen. Virol.,* **73**: 1587-1591.

Das, S. C., Michael, D. Baron, and Thomas Barrett, (2000). Recovery and Characterization of a Chimeric Rinderpest Virus with the Glycoproteins of Peste-des-Petits-Ruminants Virus: Homologous F and H proteins are required for Virus Viability.*J. Virol.* **74**: 9039-47

Davies, F.G., (1976). Characteristics of a virus causing a pox disease of sheep and goats in Kenya, with observations on the epidemiology and control. *Journal of Hygiene (Cambridge)* **76,** 163-171.

Davies, F.G., (1981). Sheep and Goat pox. In Virus diseases of Food Animals. Vol 2., E.P.J. Gibbs ed., *London Academic Press,* pp 733-748.

Davies, F.G., (1982). Observations on the epidemiology of lumpy skin disease in Kenya. *Journal of Hygiene (Cambridge),* **82,** 95-102

Davies, F.G., and Mbugwa, G., (1985). The alterations in pathogenicity of a Kenya sheep and goat pox virus on serial passage in bovine foetal muscle cell cultures. *Journal of Comparative Pathology* **95**, 565-572.

Delenda, C., Hausmann, S., Garcin, D. and Kolakofsky, D., (1997). Normal cellular replication of Sendai virus without the trans-frame, nonstructural V protein. *Virology* **228**, 55–62.

Devaux, P., Cattaneo, R., (2004). Measles virus phosphoprotein gene products: conformational flexibility of the P/V protein amino-terminal domain and C protein infectivity factor function. *J Virol.,* **78**:11632-11640.

Diallo, A., (1990). Morbillivirus group: genome organisation and proteins. *Vet. Microbiol.,* **23**, 155-163

Diallo, A., (2003). La peste des Petits Ruminants In *Principales maladies infectieuses et parasitaires du bétail Europe et régions chaudes.* (ed. Editions Tec et Doc, E. M. i.); 307-322 (Lavoisier, Paris, 2003).

Diallo, A., (2004). Control of Peste des Petits ruminants: vaccination for the control of Peste des Petits Ruminants. *Dev Biol. Basel*, **119**: 93-98

Diallo, A., Barrett, T., Lefevre, P.C. and Taylor, W.P., (1987). Comparison of proteins induced in cells infected with rinderpest and peste des petits ruminants viruses. *Journal of General Virology* **68**, 2033–8.

Diallo, A., Barrett T., Barbron, M., Subbarao, S.M. and Taylo, W.P., (1989). Differentiation of rinderpest and peste des petits ruminants viruses using specific cDNA clones. *Journal of Virological Methods* **23**(2), 127-36.

Diallo, A., Barrett, T., Barbron, M., Meyer, G. and Lefevre, P.C., (1994). Cloning of the nucleocapsid protein gene of peste-des-petits-ruminants virus: relationship to other morbilliviruses. *Journal of General Virology* **75** (Pt 1), 233–7.

Duprex, W.P., Collins, F.M. and Rima, B.K., (2002). Modulating the function of the measles virus RNA-dependent RNA polymerase by insertion of green fluorescent protein into the open reading frame. *Journal of Virology* **76**, 7322–8.

Durojaiye, O.A., Taylor, W.P. and Smale, C., (1985). The ultastructure of peste des petits ruminants virus. *Zentralblatt für Veterinärmedizin*, **32**: 460-465

Egan, M.A., Pavlat, W.A., Tartaglia, J., Paoletti, E., Weinhold, K.J., Clements, M.-L. and Siliciano, R.F., (1995). Induction of human immunodeficiency virus type 1 (HIV-1)-specific cytolytic T lymphocyte responses in seronegative adults by a nonreplicating, host-range-restricted canarypox vector (ALVAC) carrying the HIV-1MN env gene. *Journal of Infectious Diseases.* **171**, 1623–1627.

Emergency Prevention System (EMPRES) for Transboundary Plant and Animal Pests and Diseases 2000; no. 13. Available from: URL: http://www.fao.org/empres 712 Emerging Infectious Diseases • Vol. 8, No. 7, July 2002

Escoffier, C., Manie, S., Vincent, S., Muller, C. P., Billiter, M. and Gerlier, D., (1999). Nonstructural C protein is required for efficient measles virus replication in human peripheral blood cells. *Journal of Virology* **73**, 1695–8.

Evans, S.A., Belsham, G.J. and Barrett, T., (1990). The role of the 5_ nontranslated regions of the fusion protein mRNAs of canine distemper virus and rinderpest virus. *Virology* **177**, 317–23.

Evans, S.A., Baron, M.D., Chamberlain, R.W., Goatley, L. and Barrett, T., (1994). Nucleotide sequence comparisons of the fusion protein gene from virulent and attenuated strains of rinderpest virus. *Journal of General Virology* **75**, 3611–17.

Fearns, R., Peeples, M.E. and Collins, P.L., (1997). Increased expression of the N protein of respiratory syncytial virus stimulates minigenome replication but does not alter the balance between the synthesis of mRNA antigenome. *Virology* **236**, 188–201.

Finke, S. and Conzelmann, K.K., (2003). Dissociation of rabies virus matrix protein functions in regulation of viral RNA synthesis and virus assembly. *Journal of Virology* **77**, 12074–82.

Flanagan, K., Glover, R.T., Horig, H., Yang, W. and Kaufman, H.L., (2004). Local delivery of recombinant vaccinia virus expressing secondary lymphoid chemokine (SLC) results in a CD4 T-cell dependent antitumour response. *Vaccine* **22**(21-22), 2894-2903.

Fleury, B., Janvier, G., Pialoux, G., Buseyne, F., Robertson, M.N., Tartaglia, J., Paoletti, E., Kieny, M.P., Excler, J.L. and Rivière, Y., (1996). Memory cytotoxic T lymphocyte responses in human immunodeficiency virus type 1 (HIV-1)-negative volunteers immunized with a recombinant canarypox expressing gp 160 of HIV-1 and boosted with a recombinant gp160. *Journal of Infectious Diseases* **174**(4), 734-8.

Flexner, C., B. R. Murphy, J. F. Rooney, C. Wohlenberg, V. Yuferov, A. L. Notkins, and B. Moss. (1988). Succesful vaccination with a polyvalent live vector despite existing immunity to an expressed antigen. *Nature,* **335:**259-262

Franchini, G., Robert-Guroff, M., Tartaglia, J., Aggarwal, A., Abimiku, A., Benson, J., Markham, P., Limbach, K., Hurteau, G., Fullen, J., Aldrich, K., Miller, N., Sadoff, J., Paoletti, E. and Gallo, R.C., (1995). Highly attenuated HIV type 2 recombinant poxviruses, but not HIV-2 recombinant Salmonella vaccines, induce long-lasting protection in rhesus macaques. *AIDS Research in Human Retroviruses* **11**, 909–920.

Franchini, G., Tartaglia, J., Markham, P., Benson, J., Fullen, J., Wills, M., Arp, J., Dekaban, G., Paoletti, E. and Gallo, R.C., (1995). Highly attenuated HTLV type Ienv poxvirus vaccines induce protection against a cell-associated HTLV type I challenge in rabbits. *AIDS Research in Human Retroviruses* **11**, 307–313.

Fries, L.F., Tartaglia, J., Taylor, J., Kauffman, E.K., Meignier, B., Paoletti, E. and Plotkin, S., (1996). Human safety and immunogenicity of a canarypox-rabies

glycoprotein recombinant vaccine: an alternative poxvirus vector system**.** *Vaccine* **14**, 428–434.

Fries, L.F., Tartaglia, J., Taylor, J., Kauffman, E.K., Meignier, B., Paoletti, E. and Plotkin, S., (1996). Human safety and immunogenicity of a canarypox-rabies glycoprotein recombinant vaccine: an alternative poxvirus vector system**.** *Vaccine* **14**, 428–434.

Funahashi, S.; Itamura, S.; Linuma, H.; Nerome, K.; Sigimoto, M. and Shida, H., (1991). Increased expression *in vivo* and *in vitro* of foreign genes directed by A-Type Inclusion body hybrid promoters in recombinant vaccinia viruses. *Journal of Virology* **65** (10), 5584-5588.

Furley, C.W., Taylor, W.P., and Obi, T.U., (1987). An outbreak of peste des petits ruminants in a zoological collection. *The Veterinary Record*, **121**, 443-447.

Gargadennec, L. and Lalanne, A., (1942). La Peste des Petits Ruminants. *Bull. Serv. Zoot. Epiz. A. O. F.*, **5**(1):16-21

Gershon, P.D. and Black, D.N., (1987). Physical characterization of the genome of a cattle isolate of Capripoxvirus. *Virology* **160,** 473-476.

Gershon, P.D. and Black, D.N., (1989). The nucleotide sequence around the Capripoxvirus thymidine kinase gene reveals a gene shared specifically with Leporipoxvirus. *Journal of General Virology* **70**, 525-533.

Ghildyal, R., Mills, J., Murray, M., Vardaxis, N. and Meanger, J., (2002). Respiratory syncytial virus matrix protein associates with nucleocapsids in infected cells. *J. Gen. Virol.*, **83**: 753-757.

Gibbs, E.P.J., Taylor, W.P., Lawman, M.J.P. and Bryant, J., (1979). Classification of Peste des Petits Ruminants as the fourth member of the genus Morbillivirus. *Intervirology* **11**, 268-274.

Goebel, S.J., Johnson, G.P., Perkus, M.E., Davis, S.W., Winslow, J.P. and Paoletti, E., (1990). The complete DNA sequence of vaccinia virus. *Virology* **179**, 247–266.

Gombart, A.F., Hirano, A. and Wong, T.C., (1993). Conformational maturation of measles virus nucleocapsid protein. *Journal of Virology* **67**, 4133–41.

Govindarajan, R., Koteeswaran, A., Venugopalan, A.T., Shyam, G., Shagu, S., Shaila,M.S. and Ramachandran S., (1997). Isolation of peste des petits ruminants virus (PPRV) from an outbreak in Indian Buffalo (Bubalus bubalus). *Vet Rec*, **141**: 573 – 574

Greth, A., Gourreau, J.M., Vassart, M., Nguyen-Ba-Vy, Wyers, M. and Lefevre, P.C., (1992). Capripoxvirus Disease in an Arabian Oryx (Oryx leucoryx) from Saudi Arabia. *J. Wild. Dis.*, 28: 295–300.

Gubbay, O., Curran, J. and Kolakofsky, D., (2001). Sendai virus genome synthesis and assembly are coupled: a possible mechanism to promote viral RNA polymerase processivity. *Journal of General Virology* **82**, 2895–903.

Gupta, A.K., Shaji, D. and Banerjee, A.K., (2003). Identification of a novel tripartite complex involved in replication of vesicular stomatitis virus genome RNA. *Journal of Virology* **77**, 732–8.

Haffar, A., Libeau, G., Moussa, A., Cecile, M. and Diallo, A., (1999). The matrix protein gene sequence analysis reveals close relationship between peste des petits ruminants virus (PPRV) and dolphin morbillivirus. *Virus Research* **64**, 69–75.

Hafez, S.M., al-Sukayran, A., dela Cruz, D., Mazloum, K.S., al-Bokmy, A.M., al-Mukayel, A. and Amjad, A.M., (1992). Development of a live cell culture camelpox vaccine. *Vaccine* **10**(8), 533-9.

Hamdy, F.M., Dardiri, A.H., and Nduaka, O., (1976). Etiology of the stomatitis-pneumo- enteritis complex in Nigerian dwarf goats. *Canadian Journal of Comparative Medecine* **40**(3), 273-284.

Hanahan, D., (1985). Heritable formation of pancreatic β-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature* **315**, 115-122

Harty, R.N. and Palese, P., (1995). Measles virus phosphoprotein (P) requires the NH2-terminal and COOH-terminal domains for interactions with the nucleoprotein (N) but only the COOH terminus for interactions with itself. *Journal of General Virology* **76**, 2863–7.

Hel, Z., Nacsa, J., Tsai, W-P., Thornton, A., Giuliani, L., Tartaglia, J. and Franchini, G., (2002). Equivalent immunogenicity of the highly attenuated poxvirus-bases ALVAC-SIV and NYVAC-SIV vaccine candidates in SIV mac251-infected macaques. *Virology* **304,** 125-134.

Hinzman, E.E., Barr, J.N. and Wertz, G.W., (2002). Identification of an upstream sequence element required for vesicular stomatitis virus mRNA transcription. *Journal of Virology* **76**, 7632–41.

Horikami, S.M., Curran, J., Kolakofsky, D. and Moyer, S.A., (1992). Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication *in vitro*. *Journal of Virology* **66**, 4901–8.

Horikami, S.M., Smallwood, S., Bankamp, B. and Moyer, S.A., (1994). An amino-proximal domain of the L protein binds to the P protein in the measles virus RNA polymerase complex. *Virology* **205**, 540–5.

Horikami, S.M., Smallwood, S. and Moyer, S.A., (1996). The Sendai virus V protein interacts with the NP protein to regulate viral genome RNA replication. *Virology* **222** (2), 383–90.

Hu, A.H., Cattaneo, R., Schwartz, S. and Norrby, E., (1994). Role of N-linked oligosaccharide chains in the processing and antigenicity of measles virus hemagglutinin protein. *Journal of General Virology* **75**, 1043–52.

Huber, M., Cattaneo, R., Spielhofer, P., Orvell, C., Norrby, E., Messerli, M., Perriard, J.-C. and Billeter, M.A., (1991). Measles virus phosphoprotein retains the nucleocapsid protein in the cytoplasm. *Virology* **185**, 299–308.

Hunter, P., and Wallace, D., (2001). Lumpy skin disease in southern Africa: a review of the disease and aspects of control. *Journal of the South African Veterinary Association* **72**(2), 68–71.

Ichihashi, Y. and Dales S., (1973). Biogenesis of Pox viruses: relationship between a translation complex and formation of A-Type inclusions. *Virology* **51**, 297-319.

Iseni, F., Baudin, F., Garcin, D., Marq, J., Ruigrok, R. and Kolakofsky, D., (2002). Chemical modification of nucleotide bases and mRNA editing depend on hexamer or nucleoprotein phase in Sendai virus nucleocapsids. *RNA*, **8**, 1056–67.

Jackson, R.J., Ramsay, A.J., Christensen, C.D., Beaton, S., Hall, D.F. and Ramshaw, I.A., (2001). Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox. *Journal of Virology* **75:** 1205-1210.

Jahrling, P.B., Hensley, L.E., Martinez, M.J., LeDuc,J.W., Rubins, K.H., Relman, D.A. and Huggins, J.W., (2004). Exploring the potential of variola virus infection of cynomlugus macaques as a model for human smallpox. *Proceedings of the National Academy of Sciences* **101** (42), 15196-15200.

Jin, N. Y., Funahashi, S., and Shida, H., (1994). Construction of vaccinia virus A-Type inclusionbody protein, tandemly repeated mutant 7.5 *kD* protein, and hemagglutinin gene promoters support high levels of expression. *Archives of Virology* **138,** 315-330.

Kara, P.D., Afonso, C.L., Wallace, D.B., Kutish, G.F., Abolnik, C., Lu, Z., Vreede, F.T., Taljaard, L.C.F., Zsak, A., Viljoen, G.J., and Rock, D.L., (2003). Comparative sequence analysis of the South African Vaccine Strain and Two Virulent Field Isolates of Lumpy Skin Disease Virus. *Archives of Virology* **148** (7), 1335-1356.

Karaca, K., Sharma, J.M., Winslow, B.J., *et al.*, (1998). Recombinant fowlpox viruses coexpressing chicken type I IFN and Newcastle disease virus HN and F genes: influence of IFN on protective efficacy and humoral responses of chickens following in ovo or post-hatch administration of recombinant viruses. *Vaccine* **16**, 1496–503.

Kato, A., Kiyotani, K., Sakai, Y., Yoshida, T. and Nagai, Y., (1997). The paramyxovirus, Sendai virus, V protein encodes a luxury function required for viral pathogenesis. *EMBO Journal* **16**, 578–87.

Kaushik, R. and Shaila, M.S., (2004). Cellular casein kinase II-mediated phosphorylation of rinderpest virus P protein is a prerequisite for its role in replication/transcription of the genome. *Journal of General Virology* **85(**Pt 3):687-91.

Kent, SJ., Stallard, V., Corey, L., Hu, SL., Morton, WR., Gritz, L., Panicali, DL. and Greenberg, PD., (1994). Analysis of cytotoxic T lyphocyte resposes to SIV proteins in SIV-infected macaques using antigen-specific stimulation with recombinant vaccinia and fowl poxviruses. *AIDS Research in Human Retroviruses* **10**(5): 551-60

Kieny, M.P., Lathe, R., Drillien, R., Spehner, D., Skory, S., Schmitt, D., Wiktor, T., Koprowski, H. & Lecocq, J.P., (1984). Expression of rabies virus glycoprotein from a recombinant vaccinia virus**.** *Nature* **312**, 163–166.

Kitching, R.P., (1983). Progress towards sheep and goat pox vaccines. *Vaccine* **1**, 4-9.

Kitching, R.P., (1986a). Passive protection of sheep against Capripoxvirus. *Research in Veterinary Science* **41**, 247-250.

Kitching, R.P., (1986b). The control of sheep and goat pox. *Revue Scientifique et Technique de l'Office International des Epizooties* **5 (2),** 503-511.

Kitching, R.P. and Taylor, W.P., (1985). Clinical and antigenic relationship between isolates of sheep and goat pox viruses. *Tropical Animal Health and Production* **17**, 64-74

Kitching, R.P., Hammond, J.M. and Black, D.N., (1986). Studies on the major common precipitating antigen of Capripoxvirus. *Journal of General Virology* **67,** 139-148.

Kitching R.P. and Smale C., (1986). Comparison of the external dimensions of capripoxvirus isolates. *Reseacrh in Veterinary Science* **41**(3), 425-7

Kitching, R.P, Hammond, J.M. and Taylor, W.P., (1987). A single vaccine for the control of Capripox infection in sheep and goats. *Research in Veterinary Science* **42**, 53–60.

Kitching, R.P., Hammond, J.M. and Black, D.N., (1986). Studies on the major common precipitating antigen of Capripoxvirus. *Journal of General Virology* **67,** 139-148.

Kitching, R.P., Bhat, P.P. and Black, D.N., (1989). The characterisation of African strains of Capripoxvirus. *Epidemiology and Infections* **102**, 335-343.

Kolakofsky, D., Pelet, T., Garcin, D., Hausmann, S., Curran, J. and Roux, L., (1998). Paramyxovirus RNA synthesis and the requirement for hexamer genome length: the rule of six revisited. *Journal of Virology* **72**, 891–9.

Kolakofsky, D., Le Mercier, P., Iseni, F. and Garcin, D., (2004). Viral DNA polymerase scanning and the gymnastics of Sendai virus RNA synthesis. *Virology* **318**, 463–73.

Kozak, M., (1986). Point mutations define a sequence flanking the AUG initiation codon that modulates translation by eukaryotic ribosomes. *Cell*, 44: 283-292.

Kumar, S. and Boyle, DB., (1990). Activity of a fowlpox virus late gene promoter in vaccinia and fowlpox virus recombinants. *Arch Virol.* **112**(3-4):139-48.

Kundig, TM., **Kalberer, CP**., **Hengartner, H**, **and Zinkernagel, RM., (1993).** Vaccination with two different vaccinia recombinant viruses: long-term inhibition of secondary vaccination. *Vaccine*, 11: 1154-8

Kurotani, A., Kiyotani, K., Kato, A., Shioda, T., Sakai, Y., Mizumoto, K., Yoshida, T. and Nagai, Y., (1998). Sendai virus C proteins are categorically nonessential gene products but silencing their expression severley impairs viral replication and pathogenesis. *Genes to Cells* **3**, 111–24.

Kwak, H. Horig and Kaufman, H.L., (2003). Poxviruses as vectors for cancer immunotherapy, *Current Opinion in Drug Discovery and Development* **6**, 161–168.

Kwak, H., Mustafa, W., Speirs, K., Abdool, A.J., Paterson, Y. and Isaacs, S.N., (2004). Improved protection conferred by vaccination with a recombinant vaccinia virus that incorporates a foreign antigen into the extracellular enveloped virion. *Virology* **322,** 337-348.

Kwiatek, O., Minet, C., Grillet, C., Hurard, C., Carlsson, E. , Karimov, B., Albina, E., Diallo, A., and Libeau G. Peste des Petits Ruminants (PPR) Outbreak in Tajikistan (submitted for publication).

Langedijk, J., Daus, F. and Oirschot, J. von, (1997). Sequence and structure alignment of Paramyxoviridae attachment proteins and discovery of enzymatic activity for a morbillivirus hemagglutinin. *Journal of Virology* **71**, 6155–67.

Lecouturier, V., Fayolle, J., Caballero, M., Carabana, J., Celma, M. L., Fernandez-Muñoz, R., Wild, T. F. and Buckland, R., (1996). Identification of two amino acids in the hemagglutinin glycoprotein of measles virus (MV) that govern hemadsorption, HeLa cell fusion, and CD46 downregulation: phenotypic markers that differentiate vaccine and wild-type MV strains. *Journal of Virology* **70**, 4200–4.

Lefevre P.C., and Diallo A., (1990). La Peste des Petits Ruminants. *Revue Scientifique et Technique de l'Office International des Epizooties*, **9**, 935-950.

Lefevre P.C., Diallo A., Schenkel F., Hussein S. & Staak G., (1991). Serological evidence of peste des petits ruminants in Jordan. *The Veterinary Record* **128**, 110.

Lewandoski, M., (2001). Conditional control of gene expression in the mouse. *Nature Review Genetic **2,*** 743-755.

Libeau, G., Prehaud, C., Lancelot, R., Colas, F., L. Guerre, Bishop, D.H.L and Diallo A.,. (1995). Development of a competitive ELISA for peste des petits ruminants virus antibody detection using a recombinant N protein. *Res. Vet. Sci.*, **58 :** 50-55

Liermann, H., Harder,.T.C., Lochelt, M., von Messling, V., Baumgartner, W., Moennig, V. and Haas, L., (1998). Genetic analysis of the central untranslated genome region and the proximal coding part of the F gene of wild-type and vaccine canine distemper morbilliviruses. *Virus Genes* **17** (3), 259–70.

Lin, G.Y., Paterson, R.G., Richardson, C.D. and Lamb, R.A., (1998). The V protein of the paramyxovirus SV5 interacts with damage-specific DNA binding protein. *Virology* **249**, 189–200.

Liston, P., DiFlumeri, C. and Briedis, D.J., (1995). Protein interactions entered into by the measles virus P proteins, V proteins, and C proteins. *Virus Research* **38**, 241–59.

Liston, P. and Briedis, D.J., (1994). Measles virus V protein binds zinc. *Virology* **198**, 399–404.

Mackett, M., Smith, G.L. and Moss, B., (1982). Vaccinia virus: a selectable eukaryotic cloning and expression vector. *Proceedings of the National Academy of Sciences USA* **79**(23):7415-9.

Mackett, M.; Smith, G.L. and Moss, B., (1985). The construction and characterisation of vaccinia virus recombinants expressing foreign genes. In: D.M. Glover, Editor, *DNA cloning, a practical approach* IRL Press, Oxford (1985), 191–211.

Mahapatra, M., Parida, S., Egziabher, B.G., Diallo, A. and Barrett, T., (2003). Sequence analysis of the phosphoprotein gene of peste des petits ruminants (PPR) virus: editing of the gene transcript. *Virus Research* **96**, 85–98.

Maisner, A., Klenk, H.-D. and Herrler, G., (1998). Polarized budding of measles virus is not determined by viral surface glycoproteins. *Journal of Virology* **72**, 5276–8.

Malur, A.G., Choudhary, S.K., De, B.P. and Banerjee, A.K., (2002). Role of a highly conserved NH(2)-terminal domain of the human parainfluenza virus type 3 RNA polymerase. *Journal of Virology* **76**, 8101–9.

Matthews, R.E.F., (1982). Classification and nomenclature of viruses. *Intervirology* **17**,1 -99.

Martha Yami. Séquence et analyse du gène de la polymérase (L) et de la séquence extra-génique terminale du virus de la peste des petits ruminants. *Mémoire de Maîtrise -es-Sciences Vétérinaires ; Ecole Nationale vétérinaire de Toulouse*, 2001.

McIlhatton, M.A., Curran, M.D. and Rima, B.K., (1997). Nucleotide sequence analysis of the large (L) genes of phocine distemper virus and canine distemper virus (corrected sequence) *Journal of General Virology* **78**, 571–6.

Merchlinsky, M. and Moss, B., (1992). Introduction of foreign DNA into the VV genome by *in vitro* ligation: recombinant-independent selectable cloning vectors. *Virology* **190**, 522–526.

Merz, D.C., Scheid A., Choppin P.W., (1980). Importance of antibodies to the fusion glycoprotein of paramyxoviruses in the prevention of spread of infection. *J. Exp. Med.* **151**: 275-288.

Merz, D.C., Scheid A., Choppin P.W., (1981). Immunological studies of the functions of Paramyxovirus glycoproteins. *Virology* **109**: 94-105.

Meyer, G. and Diallo, A., (1995). The nucleotide sequence of the fusion protein gene of the peste des petits ruminants virus - the long untranslated region in the 5_-end of the F-protein gene of morbilliviruses seems to be specific to each virus. *Virus Research* **37**, 23–35.

Mitra-Kaushik, S., Nayak, R. and Shaila, M.S., (2001). Identification of a cytotoxic T-cell epitope on the recombinant nucleocapsid proteins of Rinderpest and Peste des petits ruminants viruses presented as assembled nucleocapsids. *Virology* **279**, 210–20.

Moll, M., Klenk, H,D., Herrler, G. and Maisner, A., (2201). A single amino acid change in the cytoplasmic domains of measles virus glycoproteins H and F alters targeting, endocytosis, and cell fusion in polarized Madin–Darby canine kidney cells. *Journal of Biologicals and Chemicals* **276**, 17887–94.

Moll, M., Klenk, H.D. and Maisner, A., (2002). Importance of the cytoplasmic tails of the measles virus glycoproteins for fusogenic activity and the generation of recombinant measles viruses. *Journal of Virology* **76**, 7174–86.

Moll, M., Pfeuffer, J., Klenk, H.D., Niewiesk, S. and Maisner, A., (2004). Polarized glycoprotein targeting affects the spread of measles virus *in vitro* and *in vivo*. *Journal of General Virology* **85**, 1019–27.

Mornet, P., Orue, J., Gilbert, Y., and collab., (1956). La Peste des Petits Ruminants en Afrique Occidentale française : ses rapports avec la Peste Bovine. *Revue d'Elevage et de Médecine des Pays Tropicaux* **9**(4), 313-342.

Moss, B., (1996). Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety, *Proceedings of the National Academy of Sciences USA* **93**, 11341–8.

Moss, B., (1996). Poxviridae: the viruses and their replication. In: Fields, B.N. Knipe, D.M. and Howley, P.M. Editors, *Fields virology*, Lippincott Raven Press, New York, 2637–2671.

Moss, B., (2001). Poxviridae: the viruses and and their replication. In Virology, 4th edn, pp. 2849–2883. Edited by D. M. Knipe & P. M. Howley. Philidelphia: Lippincott Williams and Wilkins.

Muthuchelvan, D., Sanyal, A., Singh, RP., Hemadri, D., Sen, A., Sreenivasa, BP., Singh, RK., Bandyopadhyay, SK., (2005). Comparative sequence analysis of the large polymerase protein (L) gene of peste-des-petits ruminants (PPR) vaccine virus of Indian origin. *Arch. Virol.*, **150**: 2467-2481

Munz E.K. and Owen N.C., (1966). Electron microscopic studies on Lumpy Skin Disease virus type "Neethling". Onderstepoort *Journal of Veterinary Research* **33** (1), 3-8.

Myagkikh, M., Alipanah, S., Markham, P.D., Tartaglia, J., Paoletti, E., Gallo, R.C., Franchini, G. and Robert-Guroff, M., (1996). Multiple immunizations with attenuated poxvirus HIV type 2 recombinants and subunit boosts required for protection of rhesus macaques. *AIDS Research in Human Retroviruses* **12**, 985–992.

Nacsa, J., Radaelli, A., Edghill-Smith, Y., Venzon, D., Tsai, WP., Morghen, Cde, G., Panicali, D., Tartaglia, J. and Franchini, G., (2004) Avipox-based simian immunodeficiency virus (SIV) vaccines elicit a high frequency of SIV-specific CD4+ and CD8+ T-cell responses in vaccinia-experienced SIVmac251-infected macaques. *Vaccine***,** **22**: 597-606

Naim, H.Y., Ehler, E. and Billeter, M.A., (2000). Measles virus matrix protein specifies apical virus release and glycoprotein sorting in epithelial cells. *EMBO Journal* **19**, 3576–85.

Nanda, S.K. and Baron, M.D., (2006). Rinderpest Virus Blocks Type I and Type II Interferon Action: Role of Structural and Nonstructural Proteins. *J. Virol.*, 80: 7555-7568

Ngichabe, C.K., Wamwayi, H.M., Barrett, T., Ndungu, E.K., Black, D.N., and Bostock, C.J., (1997). Trial of a capripoxvirus-rinderpest recombinant vaccine in African cattle. *Epidemiology and Infection* **118** (1), 63-70.

Office International des Epizooties (OIE) Manual, 2004. Manual of diagnostic tests and vaccines for terrestrial animals. *Fifth edition* Chapter 2.1.10, pp 211-220.

Office International des Epizooties (OIE) disease information 1999;**12**, 137.

Ohno, S., Ono, N., Takeda, M., Takeuchi, K. and Yanagi, Y., (2004). Dissection of measles virus V protein in relation to its ability to block alpha/beta interferon signal transduction. *J. Gen. Virol*., **85**: 2991 – 2999

Ozkul, A., Barrett, T., Karaoglu, T., Dagalp, SB., Yesilbag, K., Cokcaliskan, C., Gencay, A. and Burgu, I., (2002). Prevalence, distribution, and host range of Peste des petits ruminants virus, Turkey. *Emerging Infectious Diseases* **8**(7), 708-12.

Painter, G.R. and Hostetler, K.Y., (2004). Design and development of oral drugs for the prophylaxis and treatment of smallpox infection. *Trends in Biotechnology* **22** (8), 423-427.

Palosaari, H., Parisien, JP., Rodriguez, JJ., Ulane, CM., and Horvath, CM., (2003). STAT protein interference and suppression of cytokine signal transduction by measles virus V protein. *J Virol.*, **77**: 7635-7644

Panda, A., Elankumaran, S., Krishnamurthy, S., Huang, Z. and Samal, S.K., (2004). Loss of N-linked glycosylation from the hemagglutinin-neuraminidase protein alters virulence of Newcastle disease virus. *Journal of General Virology* **78**, 4965–75.

Panicali, D. and Paoletti, E., (1982). Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. *Proceedings of the National Academy of Sciences USA* **79(16),** 4927–4931.

Paoletti, E., Lipinskas, B.R., Samsanoff, C., Mercer, S. and S. Panicali, S., (1984). Construction of live vaccinia viruses using genetically engineered poxviruses: biological activity of vaccinia virus recombinants expressing the hepatitis B virus surface antigen

and the herpes simplex virus glycoprotein D. *Proceedings of the National Academy of Sciences USA* **81**, 193–197.

Paoletti, E., (1996). Applications of pox virus vectors to vaccination: an update, *Proceedings of the National Academy of Sciences USA* **93**, 11349–11353.

Pastoret, P.P., and Vanderplasschen, A., (2003). Poxviruses as vaccine vectors, *Comparative Immunology, Microbiology and Infectious Diseases* **26**, 343–355.

Paterson, R.G., Leser, G.P., Shaughnessy, M.A. and Lamb, R.A., (1995). The paramyxovirus SV5 V protein binds two atoms of zinc and is a structural component of virions. *Virology* **208**, 121–31.

Patterson, J.B., Thomas, D., Lewicki, H., Billeter, M.A. and Oldstone, B.A., (2000). V and C proteins of measles virus function as virulence factors *in vivo*. *Virology* **267**, 80–9.

Peeples, M.E., (1991). Paramyxovirus M proteins: pulling it all together and taking it on the road. In: David W. Kingsbury (ed.), *The Paramyxoviruses*. New York: Plenum Press, 427–56.

Perkus, M., Piccini, A., Lipinskas, B. and Paoletti, E., (1985). Recombinant vaccinia virus: immunization against multiple pathogens. *Science.* **229**, 981–984.

Perl, S., Alexander, A., Yakobson, B., Nyska, A., Harmelin, A., Sheikhat, N., Shimshony, A., Davidson, N., Abramson, M. and Rapoport, E., (1994). Peste des petits ruminants (PPR) of sheep in Israel: case report. *Israel Journal of Veterinary Medecine* **49**, 59–62.

Perry, B.D., Randolph, T.F., McDermott, J.J., Sones, K.R. and Thornton, P.K. Investing in animal health research to alleviate poverty. *ILRI (International Livestock Research Institute), Nairobi, Kenya,* 2002, 148 pp

Pialoux, G., Excler, J.-L., Rivière, Y., Gonzalez-Canali, G., Feuillie, V., Coulaud, P., Gluckman, J.-C., Matthews, T.J., Meignier, B., Kieny, M.-P., Gonnet, P., Diaz, I., Méric, C., Paoletti, E., Tartaglia, J., Salomon, H., Plotkin, S., The AGIS Group and L'Agence Nationale de Recherche sur le SIDA, (1995). A prime-boost approach to HIV preventive vaccine using a recombinant canarypox virus expressing glycoprotein 160 (MN) followed by a recombinant glycoprotein 160 (MN/LAI). The AGIS Group, and l'Agence Nationale de Recherche sur le SIDA. *AIDS Research in Human Retroviruses.* **11**, 373–381.

Plemper, R.K., Hammond, A.L. and Cattaneo, R., (2000). Characterization of a region of the measles virus hemagglutinin sufficient for its dimerization. *Journal of Virology* **74**, 6485–93.

Plemper, R.K., Hammond, A.L. and Cattaneo, R., (2001). Measles virus envelope glycoproteins hetero-oligomerize in the endoplasmic reticulum. *Journal of Biologicals and Chemicals* **276**, 44239–46.

Plemper, R.K., Hammond, A.L., Gerlier, D., Fielding, A.K. and Cattaneo, R., (2002). Strength of envelope protein interaction modulates cytopathicity of measles virus. *Journal of Virology* **76**, 5051–61.

Precious, B., Young, D.F., Bermingham, A., Fearns, R., Ryan, M. and Randall, R.E., (1995). Inducible expression of the P, V, and Np genes of the paramyxovirus simian virus 5 in cell lines and an examination of NP-P and NP-V interactions. *Journal of Virology* **69**, 8001–10.

Radecke, F., Spielhofer, P., Schneider, H., Kaelin, K., Huber, M., Ditsch, C., Christiansen, G. and Billeter, M.A., (1995). Rescue of measles virus from cloned DNA. *EMBO Journal* **14**, 5773–84.

Radecke, F. and Billeter, M.A., (1996). The nonstructural C protein is not essential for multiplication of Edmonston B strain measles virus in cultured cells. *Virology* **217**, 418–21.

Rahaman, A., Srinivasan, N., Shamala, N. and Shaila, M.S., (2004). Phosphoprotein of the rinderpest virus forms a tetramer through a coiled coil region important for biological function: a structural insight. *Journal of Biologicals and Chemicals* **279** (22), 23606–14.

Rager, M., **Vongpunsawad, S.**, **Duprex, W.P.**, **Cattaneo, R.** (2002). Polyploid measles virus with hexameric genome length. *EMBO J.* **15** (10):2364-72

Ramírez, J.C., Magdalena Gherardi,, M., Dolores Rodríguez, and Mariano Esteban, (2000). Attenuated Modified Vaccinia Virus Ankara Can Be Used as an Immunizing Agent under Conditions of Preexisting Immunity to the Vector. *J. Virol.*, 74: 7651-7655.

Ramyar, H. and Hessami, M., (1967). Development of an attenuated live virus vaccine against sheep pox. *Zentralbl Veternaremed B.* **14**(6), 516-9

Ramyar, H., (1965). Studies on the immunogenic properties of tissue culture sheep pox virus. *Zentralbl. Veterinarmed,* **123,** 537-540.

Randall, R.E. and Bermingham, A., (1996). NP-P and NP-V interactions of the paramyxovirus simian virus 5 examined using a novel protein-protein capture assay. *Virology* **224**, 121–9.

Redfield, R. R., Wright, D. C., James, W. D., Jones, T. S., Brown, C. and Burke, D. C., (1987). Disseminated vaccinia in military recruit with human immunodeficiency virus (HIV) disease. *New England Journal of Medicine* **316**, 673–676.

Renshaw, H.W., and Dodd, A.G., (1978). Serological and crossimmunity studies with contagious ecthyma and goat pox viruses isolated from the Western United States. *Archives of Virology* **56**, 201-210.

Reuter, T., B. Weissbrich, S. Schneider-Schaulies, and Schneider-Schaulies, J., (2006). RNA Interference with Measles Virus N, P, and L mRNAs Efficiently Prevents and with Matrix Protein mRNA Enhances Viral Transcription. *J. Virol*., **80**: 5951-5957.

Riedl, P., Moll, M., Klenk, H.D. and Maisner, A., (2002). Measles virus matrix protein is not cotransported with the viral glycoproteins but requires virus infection for efficient surface targeting. *Virus Research* **83**, 1–12.

Rima, B.K., Baczko, K., Clarke, D.K., Curran, M.D., Martin, S.J., Billeter, M.A. and Ter Meulen, V., (1986). Characterization of clones for the sixth (L) gene and transcriptional map for morbilliviruses. *J. Gen. Virol.*, **67:** 1971-1978.

Rima, BK, Collin, AM, and Earle, JA., (2005). Completion of the sequence of a cetacean morbillivirus and comparative analysis of the complete genome sequences of four morbilliviruses. *Virus Genes*, 30:113-9.

Rodriguez-Boulan, E. and Nelson, W.J., (1989). Morphogenesis of the polarized epithelial cell phenotype. *Science* **245**, 718–25.

Roeder, P.L., Abraham, G., Kinfe, G. and Barrett, T., (1994). Peste des Petits Ruminants Virus in Ethiopian goats. *Tropical Animal Health and Production*. **26**, 69-73.

Roger, F., Yigezu, L.M., Hurard, C., Libeau, G., Mebratu, G.Y., Diallo, A. and Faye, B.,(2000). Investigations on a new pathological condition of camels in Ethiopia. *J. Camel Practice and Res*., 2000, **7**: 163-165.

Roger, F., Gebre, Yesus M., Libeau, G., Diallo, A., Yigezu, L.M. and Yilma, T., (2001). Detection of antibodies of rinderpest and peste des petits ruminants viruses (*Paramyxoviridae, Morbillivirus*) during a new epizootic disease in Ethiopian camels (*Camelus dromadarius*). *Rev. Méd. Vét.*, **152:** 265-268.

Rolph, M.S. and Ramshaw, I.A., (1997), Recombinant viruses as vaccines and immunological tools, *Current Opinion Immunology* **9**, 517–524.

Romero, C.H., Barrett, T., Evans, S.A., Kitching, R.P., Gershon, P.D., Bostock, C. and Black, D.N., (1993). Single capripox virus recombinant vaccine for the protection of cattle against rinderpest and lumpy skin disease. *Vaccine* **11**, 737-742.

Romero, C.H., Barrett, T., Chamberlain, R.W., Kitching, R.P., Fleming, M., and Black, D.N., (1994a). Recombinant capripox expressing the hemagglutin protein gene of rinderpest virus protection of cattle against rinderpest and lumpy skin disease viruses. *Virology* **204,** 425-429.

Romero, C.H., Barrett, T., Kitching, R.P., Carn, V.M., and Black, D.N., (1994b). Protection of cattle against rinderpest and lumpy skin disease with a recombinant capripox expressing the fusion protein gene of rinderpest virus. *The Veterinary Record* **135,** 152-154.

Romero, C.H., Barrett, T., Kitching, R.P., Bostock, C. and Black, D.N., (1995). Protection of goats against *peste des petits ruminants* with recombinant capripoxviruses expressing the fusion and haemagglutinin protein genes of rinderpest virus. *Vaccine* **44**, 151–163.

Rooney, J.F., Wohlenberg, C., Cremer, K. J., Moss, B., and Notkins, A. L., (1988). Immunization with a vaccinia virus recombinant expressing herpes simplex virustype 1 glycoprotein D: long-term protection and effect of revaccination. *J. Virol.,* **62:**1530-1534

Ryan, K.W. and Portner, A., (1990). Separate domains of Sendai virus P protein are required for binding to viral nucleocapsids. *Virology* **174**, 515–21.

Sabban, M.S., (1957). The cultivation of sheep pox virus on the chorioallantoic membrane of the developing chicken embryo. *American Journal of Veterinary Research* **18**(68), 618-24

Samal, S.K. and Collins, P.L., (1996). RNA replication by a respiratory sycytial virus analog does not obey the rule of six and retains a nonviral trinucleotide extension at the leader end. *Journal of Virology* **70**, 5075–82.

Sato, H., Masuda, M., Miura, R., Yoneda, M., Kai, C., (2006). Morbillivirus nucleoprotein possesses a novel nuclear localization signal and a CRM1-independent nuclear export signal. *Virology*, 352: 121-30

Schramm, B., Locker, J.K., (2005). Cytoplasmic organization of Poxvirus DNA replication. *Traffic* **6,** 839-846.

Schneider, H., Kaelin, K. and Billeter, M.A., (1997). Recombinant measles viruses defective for RNA editing and V protein synthesis are viable in cultured cells. *Virology,* **227**, 314–22.

Scott G., (1981). Rinderpest and Peste des Petits Ruminants. *Virus diseases of food animals. Vol II. Gibbs E.P.J., Londres, Acad. Press,* 402-432.

Seth, S. and Shaila, M.S., (2001). The hemagglutinin-neuraminidase protein of peste des petits ruminants virus is biologically active when transiently expressed in mammalian cells. *Virus Research* **75**, 169–77.

Shaffer, JA., Bellini, WJ., Rota, PA., (2003). The C protein of measles virus inhibits the type I interferon response. *Virology,* **315**: 389-97.

Shaji, D. and Shaila, M.S., (1999). Domains of rinderpest virus phosphoprotein involved in interaction with itself and the nucleocapsid protein. *Virology,* **258**, 415–24.

Shaila, M.S., Purushothaman, V., Bhavasar, D., Venugopal, K. and Venkatesan, R.A., (1989). Peste des petits ruminants of sheep in India. *Vet. Rec.*, **125**: 602.

Shaila, MS., Shamaki, D., Forsyth, MA., Diallo, A., Goatley, L., Kitching, RP., Barrett, T., (1996a). Geographic distribution and epidemiology of peste des petits ruminants virus. *Virus Research* **43**(2), 149-53.

Shaila, M.S., Peter, A.B., Varalakshmi, P., Apte, M., Rajendran, M.P. and Anbumani, S.P. (1996b). Peste des petits ruminants in Tamilnadu goats. *Indian Vet. J.,* **73**: 587-588

Shida, H.; Tanabe, K. and Matsumoto, S., (1977). Mechanism of virus occlusion in to A-Type inclusion during poxvirus infection. *Virology,* **76**: 217-233

Sidhu, MS., Husar, W., Cook, SD., Dowling, PC. and Udem, SA., (1993). Canine distemper terminal and intergenic non-protein coding nucleotide sequences: completion of the entire CDV genome sequence. *Virology*, 193:66-72.

Smallwood, S., Ryan, K.W. and Moyer, S.A., (1994). Deletion analysis defines a carboxylproximal region of Sendai virus P protein that binds to the polymerase L protein. *Virology* **202**, 154–63.

Smith, G.L., Mackett, M., and Moss, B., (1983). Infectious vaccinia virus recombinants that express hepatitis B virus surface antigen. *Nature* **302**, 490–5.

Smith, G.L. and Moss, B., (1983). Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA. *Gene* **25,** 21–28.

Smith, G.L.; Cheng, K.C and Moss, B., (1986). Vaccinia virus: an expression vector for genes from parasites, *Parasitology*, **92**, S109–S117.

Spehner, D., Drillien, R. and Howley, P.M., (1997). The assembly of the measles virus nucleoprotein into nucleocapsid- like particles is modulated by the phosphoprotein. *Virology* **232**, 260–8.

Stannard, L.M., Marais, D.M, Kow, D., and Dumbell, K.R., (1998). Evidence for incomplete repication of a penguin poxvirus in cells of mammalian origin. *Journal of General Virology* **79,** 1637–46.

Stern, L., M. Greenberg, J. M. Gershoni, and S. Rozenblatt., (1995). The hemagglutinin envelope protein of canine distemper virus (CDV) confers cell tropism as illustrated by CDV and measles virus complementation analysis. *J. Virol.*, **69:**1661-1668

Stillman, E.A. and Whitt, M.A., (1998). The length and sequence composition of vesicular stomatitis virus intergenic regions affect mRNA levels and the site of transcript initiation. *Journal of Virology* **72**, 5565–72.

Stillman, E.A. and Whitt, M.A., (1999). Transcript initiation and 5_-end modifications are separable events during vesicular stomatitis virus transcription. *Journal of Virology* **73**, 7199–209.

Sugiyama, M., Ito, N., Minamoto, N. and Tanaka, S., (2002). Identification of immunodominant neutralizing epitopes on the hemagglutinin protein of rinderpest virus. *Journal of Virology* **76**, 1691–6.

Suryanarayana, K., Baczko, K., ter Meulen, V. and Wagner, R.R., (1994). Transcription inhibition and other properties of matrix proteins expressed by M genes cloned from measles viruses and diseased human brain tissue. *Journal of Virology* **68**, 1532–43.

Sutter, G. and Staib, C., (2003). Vaccinia vectors as candidate vaccines: the development of modified vaccinia virus Ankara for antigen delivery, *Current Drug Targets for Infectious Disorders* **3**, 263–271.

Sutter, G., Wyatt, LS., Foley, PL., Bennink, JR. and Moss, B., (1994). A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. *Vaccine*, 12:1032-40.

Sweetman, D.A., Miskin, J. and Baron, M.D. (2001). Rinderpest virus C and V proteins interact with the major (L) component of the viral polymerase. *Virology* **281**, 193–204.

Takeuchi, K., Kadota, S., Takeda, M., Miyajima, N., and Nagata, K., (2003). Measles virus V protein blocks interferon (IFN)-alpha/beta but not IFN-gamma signalling by inhibiting STAT1 and STAT2 phosphorylation. *FEBS Lett.*, **545:**177-182.

Takeuchi, K., Takeda, M., Miyajima, N., Ami, Y., Nagata, N., Suzaki, Y., Shahnewaz, J., Kadota, S. and, Nagata, K., (2005). Stringent requirement for the C

protein of wild-type measles virus for growth both in vitro and in macaques. *J Virol.*, **79**:7838-7844

Tantawi, H.H. and Al Falluji, M.M., (1979). Laboratory characteristics of four strains of goatpox virus. *Acta Virologica* **23**(6), 455-60

Tapparel, C. and Roux, L., (1996). The efficiency of Sendai virus genome replication: the importance of the RNA primary sequence independent of terminal complementarity. *Virology*, **225**:163-171.

Tapparel, C., Hausmann, S., Pelet, T., Curran, J., Kolakofsky, D. and Roux, L., (1997). Inhibition of Sendai virus genome replication due to promoter-increased selectivity: a possible role for the accessory C proteins. *Journal of Virology* **71**, 9588–99.

Tapparel, C., Maurice D., and Roux L., (1998). The activity of Sendai virus genomic and antigenomic promoters requires a second element past the leader template regions: a motif $(GNNNNN)_3$ is essential for replication. *J. Virol*., 1998, **72**: 3117-3128

Tartaglia, J., Perkus, M.E., Taylor, J., Norton, E.K., Audonnet, J.-C., Cox, W.I., Davis, S.W., VanderHoeven, J., Meignier, B., Rivière, M., Languet, B. and Paoletti, E., (1992). NYVAC: a highly attenuated strain of vaccinia virus. *Virology* **188**, 217–232.

Tartaglia, J., Jarrett, O., Neil, J.C., Desmettre, P. and Paoletti, E., (1993). Protection of cats against feline leukemia virus by vaccination with a canarypox virus recombinant, ALVAC-FL. *Journal of Virology* **67**, 2370–2375.

Tatsuo, H., Ono, N., and Yanagi, Y., (2001). Morbilliviruses use signaling lymphocyte activation molecules (CD150) as cellular receptors. *J. Virol*., **75:** 5842-5850

Taylor, J., Weinberg, R., Kawaoka, Y., Webster, RG. and Paoletti, E., (1988). Protective immunity against avian influenza induced by a fowlpox virus recombinant. *Vaccine* **6** (6): 504-8.

Taylor, J., Weinberg, R., Tartaglia, J., Richardson, C., Alkhatib, G., Briedis, D., Appel, M., Norton, E. and Paoletti, E., (1992). Nonreplicating viral vectors as potential vaccines: recombinant canarypox virus expressing measles virus fusion (F) and hemagglutinin (HA) glycoproteins. *Virology* **187**, 321–328.

Taylor, J. and Paoletti, E., (1988). Fowlpox virus as a vector in non-avian species. *Vaccine* **6**, 466–468.

Taylor, J., Weinberg, R., Languet, B., Desmettre, P. and Paoletti, E., (1988). Recombinant fowlpox virus inducing protective immunity in non-avian species. *Vaccine* **6**, 497–503.

Taylor, J., Trimarchi, C., Weinberg, R., Languet, B., Guillemin, F., Desmettre, P. and Paoletti, E., (1991). Efficacy studies on a canarypox-rabies recombinant virus. *Vaccine* **9**, 190–193.

Taylor, J., Meignier, B., Tartaglia, J., Languet, B., VanderHoeven, J., Franchini, G., Trimarchi, C. and Paoletti, E., (1995). Biological and immunogenic properties of a canarypox-rabies recombinant, ALVAC-RG (vCP65) in non-avian species. *Vaccine* **13**, 539–549.

Taylor, J., Tartaglia, J., Rivière, M. and Paoletti, E., (1994). Applications of canarypox (ALVAC) vectors in human and veterinary vaccination. *Development of Biological Standards* **82**, 131–135.

Taylor, W.P., (1984). The distribution and epidemiology of peste des petits ruminants. *Prev. Vet. Med.*, **2**, 157-166

Taylor, W.P., Albusaidy, S., and Barrett, T., (1990). The epidemiology of pest des petits ruminants in the sultanate of Oman. *Veterinary Microbiology* **22**, 341- 352.

Taylor, WP., Diallo, A., Gopalakrishna, S., Sreeramalu, P., Wilsmore, AJ., Nanda, YP., Libeau, G., Rajasekhar, M. and Mukhopadhyay, AK., (2002). Peste des petits

ruminants has been widely present in southern India since, if not before, the late 1980s**.** *Prev. Vet. Med.*, **52**:305-312

Tober, C., Seufert, M., Schneider, H., Billeter, M.A., Johnston, I.C.., Niewiesk, S., ter Meulen, V. and Schneider-Schaulies, S., (1998). Expression of measles virus V protein is associated with pathogenicity and control of viral RNA synthesis. *Journal of Virology,* **72**, 8124–32.

Tripathy, DN. and Wittek, R., (1990). Regulation of foreign gene in fowlpox virus by a vaccinia virus promoter, *Avian Dis*., 34:218-20.

Tulman E.R., Afonso C.L., Lu Z., Zsak L., Kutish G.F. and Rock D.L., (2001). Genome of lumpy skin disease virus. *Journal of Virology* **75**, 7122–30.

Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Sur, J-H., Sandybaev, N.T., Kerembekova, U.Z., Zaitsev, G.F., Kutish, G.F., and Rock, D.L., (2002). The genomes of sheepoox and goatpox viruses. *Journal of Virology* **76**, 6054-6061.

Valsamakis, A., Schneider, H., Auwaerter, P.G., Kaneshima, H., Billeter, M.A. and Griffin, D.E., (1998). Recombinant measles viruses with mutations in the C, V, or F gene have altered growth phenotypes *in vivo*. *Journal of Virology* **72**, 7754–61.

van Oirschot, JT., (1999). DIVA vaccines that reduce virus transmission. *Biotechnol.*, 73:195-205

Vazquez-Blomquist, D., Gonzalez S. and Duarte, C.A., (2002). Effect of promoters on cellular immune response induced by recombinant fowlpox virus expressing multi-epitope polypeptides from HIV-1. *Biotechnology and Applied Biochemistry* **36 (3),** 171-179

von Messling, V., Zimmer, G., Herrler, G., Haas, L. and Cattaneo, R., (2001). The hemagglutinin of canine distemper virus determines tropism and cytopathogenicity. *Journal of Virology* **75**, 6418–27.

von Messling, V., Svitek, N. and Cattaneo, R., (2006). Receptor (SLAM [CD150]) recognition and the V protein sustain swift lymphocyte-based invasion of mucosal tissue and lymphatic organs by a morbillivirus. *J Virol*., **80**: 6084-6092

Vongpunsawad, S., Oezgun, N., Braun, W. and Cattaneo, R., (2004). Selectively receptorblind measles viruses: Identification of residues necessary for SLAM- or CD46-induced fusion and their localization on a new hemagglutinin structural model. *Journal of Virology* **78**, 302–13.

Wade-Evans, A.M., Romero, C.H., Mellor, P., Takamatsu, H., Anderson, J., Thevasagayam, J., Fleming, M.J., Mertens, P.P.C., and Black, D.N., (1996). Expression of the major core structural protein (VP7) of bluetongue virus, by a recombinant capripoxvirus, provides partial protection of sheep against a virulent heterotypic bluetongue virus challenge. *Virology* **220**, 227-231.

Wallace, DB., Ellis, CE., Espach, A., Smith, SJ., Greyling, RR. and Viljoen GJ., (2006). Protective immune responses induced by different recombinant vaccine regimes to Rift Valley fever. *Vaccine*, in press.

Watanabe, M., Wang, A., Sheng, J., Gombart, AF., Ayata, M., Ueda, S., Hirano, A. and Wong, TC., (1995a). Delayed activation of altered fusion glycoprotein in a chronic measles virus variant that causes subacute sclerosing panencephalitis.*J Neurovirol*. **1**(2):177-88. Corrected and republished in: *J*:*Neurovirol*.,**1**:412-423

Watanabe, M., Hirano, A., Stenglein, S., Nelson, J., Thomas, G. and Wong, T.C., (1995b). Engineered serine protease inhibitor prevents furin-catalyzed activation of the fusion glycoprotein and production of infectious measles virus. *Journal of Virology* **69**, 3206–10.

Watanabe, N., Kawano, M., Tsurudome, M., Kusagawa, S., Nishio, M., Komada H., Shima, T. and Ito, Y., (1996). Identification of the sequences responsible for nuclear targeting of the V protein of human parainfluenza virus type 2. *Journal of General Virology* **77** (Pt 2), 327–38.

Welter, J., Taylor, J., Tartaglia, J., Paoletti, E. and Stephenson, C., (2000). Vaccination against canine distemper virus infection in infant ferrets with and without maternal antibody protection, using recombinant attenuated poxvirus vaccines. *Journal of Virology* **74**, 6358–6367.

Wertz, G.W., Perepelitsa, V.P. and Ball, L.A., (1998). Gene rearrangement attenuates expression and lethality of a nonsegmented negative strand RNA virus. *Proceedings of the. National Academy of Science USA* **95**, 3501–6.

Wertz, G.M.R. and Ball, L.A., (2002). Adding genes to the RNA genome of VSV: positionaleffects on stability of expression. *Journal of Virology* **76**, 7642–50.

Whelan, S. P. J. and Wertz, G. W., (1999). Regulation of RNA Synthesis by the Genomic Termini of Vesicular Stomatitis Virus: Identification of Distinct Sequences Essential for Transcription but Not Replication. *J. Virol.*, **73**: 297-306

Whelan, SP., Varr, JN. and Wertz, GW., (2004). Transcription and replication of nonsegmented negative-strand RNA viruses. *Curr Top Microbiol Immunol.*, **283**: 61-119

Wild T. F., Malvoisin E. and Buckland R., (1991). Measles virus: both the hemagglutinin and fusion glycoproteins are required for fusion. *J. Gen. Virol*., 72: 439-42

Willey, R., Byrum, R., Piatak, M. and 8 other authors, (2003). Control of viremia and prevention of SHIV-induced disease in rhesus macaques immunized with recombinant VV plus inactivated SIV and HIV-1 particles. *Journal of Virology* **77**, 1163–1174.

WHO (World Health Organization)., (1980). The Global Eradication of Smallpox. Final Report of the Global Commission for the Certification of Smallpox Eradication. History of International Public Health, no. 4. Geneva: World Health Organization.

Worrwall, E.E., Litamoi, J.K., Seck, B.M., and Ayelet, G., (2001). Xerovac: an ultra rapid method for the dehydration and preservation of live attenuated rinderpest and peste des petits ruminants vaccines. *Vaccine*, **19**: 834-839.

Wosu L.O., (1985). Agglutination of red blood cells by peste des petits ruminants (PPR) virus. *Nigeria Veterinary Journal*, **14**: 56-58

Wosu, L.O., (1991). Haemagglutination test for diagnosis of peste des petits ruminants disease in goats with samples from live animals. *Small ruminants Research*, **5:** 169-171.

Wu, X., Gong, X., Foley, H.D., Schnell, M.J. and Fu, Z.F., (2002). Both viral transcription and replication are reduced when the rabies virus nucleoprotein is not phosphorylated. *Journal of Virology* **76**, 4153–61.

Yang, J., Koprowsgi, H., Dietzschold, B. and Fu, Z.F., (1999). Phosphorylation of rabies virus nucleoprotein regulates viral RNA transcription and replication by modulating leader encapsidation. *Journal of Virology* **73**, 1661–4.

Yeruham, I., Nir, O., Braverman, Y., Davidson, M., Grinstein, H., Haymovitch, M., and Zamir, O., (1995). Spread of lumpy skin disease in Israeli dairy herds. *The Veterinary Record*, **137**, 91-93.

Yoshida, E., Shin, Y.S., Iwatsuki, K., Gemma, T., Miyashita, N., Tomonaga, K., Hirayama, N., Mikami, T. and Kai, C., (1999). Epitopes and nuclear localization analyses of canine distemper virus nucleocapsid protein by expression of its deletion mutants. *Veterinary Microbiology* **66**, 313–20.

Zavala, F., Rodrigues, M., Rodriguez, D., Rodriguez, J., Nussenzweig, R. and Esteban, M., (2001). A striking property of recombinant poxviruses: efficient inducers of *in vivo* expansion of primed CD8+ T cells. *Virology* **280**, 155–159.