UNIVERSITÀ DI PARMA

UNIVERSITA' DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN "SCIENZE MEDICO-VETERINARIE"

CICLO XXXII

Bovine Herpesvirus-4 vectored-based vaccine for Ovine Rinderpest

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Anni Accademici 2016/2017 – 2018/2019

The research reported in this PhD thesis has been published in an international scientific journal:

Bovine Herpesvirus-4-Based Vector Delivering Peste des Petits Ruminants Virus Hemagglutinin ORF Induces both Neutralizing Antibodies and Cytotoxic T Cell Responses

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Front Immunol . 2018 Mar 5;9:421. doi: 10.3389/fimmu.2018.00421. eCollection 2018.

Abstract

Ovine Rinderpest is an endemic infectious disease, aslo globally known as Peste des Petits Ruminants (PPR), affecting both wild type and domestic small ruminants. PPR has first appeared in West Africa in the 1940s, and since then it has been spreading across North and East Africa, and to the Near and Middle East, reaching the South and East Asia, where it has been reported in China in 2007. More recently, in 2016, it has also emerged in Europe. PPR is caused by a virus belonging to the *Morbillivirus* genus (*Paramyxoviridae* family), named Peste des Petits Ruminants Virus (PPRV), a negative single stranded RNA-based virus, primarly targeting goats and sheep. Fever, oral lesions, diarrhea, pneumonia and, often, death are all PPR characteristic symptons manifested by affected animals. The disease is thus particularly concerning for underdeveloped countries, whose economy is mainly based on small ruminants livestock, and where PPR causes drastic economic losses. In order to eradicate the disease, the Office International des Epizooties (OIE) (http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2018/) and the Food and Agriculture Organization of the United Nations (FAO) have jointly developed a Global Strategy for PPR control and eradication, expected to be reached by 2030. Vaccination represents the most effective strategy for PPR control, however to date, all the commercially available live attenuated vaccines are constrained by thermolability and inability of Differentiating the Infected from Vaccinated Animals (DIVA), thus not being employable for sero-surveillance in an eradication program. A suitable vaccine, should instead, be thermo-stable and allow the distinction between naturally infected and vaccinated animals.

This PhD research project attempts to develop a new generation DIVA vaccine based on a recombinant Bovine herpesvirus type 4 (BoHV-4) expressing the Peste des Petit Ruminants Virus Hemagglutinin antigen (PPRV-H), capable of inducing a specific anti-PPRV-H antibody response. Viral vectors represent promising tools for gene therapy and vaccines, enhancing transgenes immunogenicity without the need of adjuvants and strongly stimulating both humoral and cell-mediated immune responses. The BoHV-4 strain employed in this PhD experiments, owns several biological and molecular characteristics that highlight it as a potential and efficient viral vector for transgenes expression, with no proof of pathogenicity or transforming activity. Since the H antigen represents PPRV immunodominant envelope glycoprotein, displays hemagglutinin and neuraminidase activities and plays a crucial role in virus attachment and penetration, it has been chosen as the transgene candidate for this DIVA vaccine. The recombinant BoHV-4 (BoHV-4-A-

PPRV-H-ΔTK) has been *in vitro* generated and *in vivo* assessed for both cellular and humoral immune responses in immunocompetent C57BL/6 mice. BoHV-4-A-PPRV-H-ΔTK inoculated mice efficiently produced specific T lymphocytes, comprehensive of $CD4^{+,}$ $CD8^{+}$ and Cytotoxic (CTL) T Limphocytes, and high titer of sero-neutralizing antibodies against PPRV-H. Helper $CD4^{+}$ T cells play a crucial immune role in presenting viral epitopes to plasma B cells while $CD8^{+}$ and CTL cytotoxic activity assure virus clearance by directly eliminating the infected cells. Although antibodies are considered the most likely effectors for inducing immune protection, studies on Rinderpest morbillivirus (RPV) showed that antibodies alone are not able to infer protection, suggesting that cell mediated immune activity intertwined with the production of high sero-neutralizing antibodies titers might ensures a protective barrier against PPRV. Thus, these positive preliminary data obtained on mice models, if further confirmed on PPRV natural hosts, suggest that recombinant BoHV-4-A-PPRV-H-ΔTK vector might represent a potential, effective vaccine candidate employable on field, in an eradication program to protect against PPRV herd infection.

Riassunto

Ovine Rinderpest, altrimenti conosciuta a livello mondiale come Peste des Petits Ruminants (PPR), è una malattia infettiva endemica in grado di colpire i piccoli ruminanti domestici e selvatici. La prima manifestazione della malattia PPR risale agli anni ’40 in Africa Occidentale, successivamente venne identificata anche in Africa Settentrionale ed Orientale e nel Vicino e Medio Oriente, raggiungendo il Sud-Est Asiatico, dove nel 2007 venne identificata in Cina. Più recentemente, nel 2016, la malattia si è manifestata anche in Europa. L’agente eziologico della PPR è un virus appartenente al genere *Morbillivirus* (famiglia *Paramyxoviridae*), definito virus Peste des Petits Ruminants (PPRV). PPRV è un virus a singolo filamento di RNA a polarità negativa, avente come target capre e pecore, nei quali induce sintomi caratteristici, quali: febbre, lesioni del cavo orale, dissenteria e polmonite, con elevata mortalità. La malattia rappresenta quindi una emergenza nei paesi in via di sviluppo la cui economia è principalmente basata sull’allevamento di ovini e caprini e dove PPR causa importanti perdite economiche. Al fine di eradicare la malattia, l’Office International des Epizooties (OIE) (http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2018/) e la Food and Agriculture Organization of the United Nations (FAO) hanno hanno sviluppato in maniera congiunta una strategia globale per il controllo e l'eradicazione della PPR, che dovrebbe essere raggiunta entro il 2030. La vaccinazione rappresenta la strategia più efficace per controllare la diffusione della malattia, tuttavia ad oggi, tutti i vaccini vivi attenuati commercialmente disponibili contro PPRV sono limitati dalla termolabilità e dalla incapacità di distinguere tra gli animali naturalmente infettati dal patogeno e quelli vaccinati (Differentiating the Infected from Vaccinated Animals, DIVA), quindi non sono impiegabili in un programma di siero-sorveglianza ed eradicazione. Un vaccino ideale dovrebbe invece essere termostabile e permettere la distinzione degli animali naturalmente infettati da quelli vaccinati.

L’obiettivo di questo progetto di Dottorato è quello di cercare di generare un vaccino DIVA di nuova generazione basato su un virus Bovine herpesvirus type 4 (BoHV-4) ricombinante, esprimente la proteina emoagglutinina del virus della Peste des Petit Ruminants (PPRV-H) ed in grado di indurre una specifica risposta umorale contro l’antigene PPRV-H. I vettori virali, capaci di incrementare l’immunogenicità dei transgeni veicolati senza la necessità di adiuvanti e di stimolare una forte risposta immunitaria sia umorale che cellulo-mediata, rappresentano strumenti promettenti per l’immunoprofilassi e la terapia genica. Il ceppo BoHV-4 utilizzato nella sperimentazione di questo progetto di Dottorato possiede diverse

caratteristiche biologiche e molecolari che lo rendono un potenziale efficiente vettore virale per l'espressione di transgeni in assenza di patogenicità o di attività trasformante.

Inoltre, poiché l'antigene H rappresenta la glicoproteina di superficie immunodominante del virus PPR, coinvolta nelle fasi di adesione e penetrazione del virus nella cellula ospite, è stato scelto come transgene candidato per questo vaccino DIVA. Il BoHV-4 ricombinante (BoHV-4-A-PPRV-H-ΔTK) è stato generato *in vitro* e validato *in vivo* per la capacità di stimolare sia la produzione di anticorpi che l'attività cellulo-mediata da parte del sistema immunitario in topi C57BL/6 immunocompetenti. I topi inoculati con BoHV-4-A-PPRV-H-ΔTK hanno efficientemente prodotto linfociti T $CD4^+$ helper e $CD8^+$ citotossici ed elevati titoli di anticorpi sieroneutralizzanti specifici contro l'antigene PPRV-H. I linfociti T $CD4^+$ helper giocano un ruolo cruciale nella presentazione degli epitopi virali alle plasmacellule B mentre l'attività citotossica dei linfociti $CD8^+$ and CTL assicura la clearance virale tramite diretta eliminazione delle cellule infettate. Sebbene gli anticorpi sieroneutralizzanti siano considerati i principali componenti immunitari in grado di fornire protezione, studi recenti su virus Riderpest (RPV) hanno mostrato che i soli anticorpi non sono in grado di indurre protezione immunitaria, suggerendo che l'attività cellulo-mediata insieme alla produzione di elevati titoli di anticorpi sieroneutralizzanti possano fornire ed assicurare una barriera protettiva contro PPRV. La potenziale conferma di questi dati sperimentali preliminari, ottenuti sul modello animale murino, nell'animale ospite del PPRV, suggerirebbe il vettore ricombinante BoHV-4-A-PPRV-H-ΔTK come possibile candidato vaccino contro la malattia PPR, impiegabile in un programma di eradicazione su campo per proteggere le mandrie di ovini e caprini dalla infezione di PPRV.

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BOVINE HERPESVIRUS TYPE 4

Bovine herpesvirus type 4 (BoHV-4) is a double stranded DNA (dsDNA) virus belonging to the *Herpesviridae* family, *Rhadinovirus* genus (or γ_2 group) and *Gammaherpesvirinae* subfamily (Zimmermann et al. 2001). BoHV-4 has been originally, incorrectly, classified as a β-herpesvirus based on its cytomegaloviruses-like cytopathogenic effects of high-density inclusion bodies and giant cells generation during tissue culture cells infection (Gaetano Donofrio et al. 2002). However, molecular analyses have made it possible for the International Committee for the Taxonomy of Viruses, to correctly reclassify BoHV-4 as γ-herpesvirus, based on its Herpesvirus Saimiri (HVS)-like B-type genome structure and γ-herpesviruses homologous genes (such as Thymidine Kinase (TK) encoding gene) presence and arrangement. (Zimmermann et al. 2001) γ-herpesviruses usually replicate in lymphocytes, and differently from α- and β-herpesviruses, they preferentially establish latent infections into either B or T cells. In addition to conserved genes among herpesviruses, each γ-herpesvirus possesses a unique genes set, usually located at genome termini that are nvolved into viral pathogenesis. Moreover γ-herpesviruses possess more cellular homologous genes respect to α- and β-herpesviruses, and are further subdivided into Lymphocryptovirus, Macavirus, Percavirus and Rhadinovirus genera (Riaz, Murtaz-ul-Hasan, e Akhtar 2017).

BoHV-4 is worldwide spread; in 1966, Bartha et al. have been the first to isolate it in Europe from a cattle affected by ocular and respiratory diseases (Bartha, Juhász, e Liebermann 1966), while in USA the first BoHV-4 isolation has been achieved in 1971 by Mohanty et al. from a 1.5-year-old bovine showing respiratory disease clinical signs (Mohanty, Hammond, e Lillie 1971). Since then, over fourty other strains have subsequently been isolated across the world, showing BoHV-4 biological and epidemiological variability (Morán et al. 2015a; Gagnon et al. 2017). Despite the high genetic relatedness among BoHV-4 isolates, genome restriction maps, based on *HindIII*, *BamHI* and *EcoRI* restriction enzymes cleavage, allowed BoHV-4s classification into three groups: the european Movar 33/63, the american DN 599 and the african buffalo strains. The european strains diverged from the american nearly 260 thousand years ago and the first BoHV-4 strain to be entirely sequenced has been the american 66-p-347 strain (B. Dewals 2006; Zimmermann et al. 2001).

Although it has been mainly isolated from cattle (*Bos taurus*), BoHV-4 susceptible species range is quite broad. Among ruminants, it has been identified in sheep (*Ovis aries*), goats

(*Capra hircus*), zebu (*Bos indicus*) (Moreno-Lopez et al. 1989; Kálmán e Egyed 2005), American bison *(Bison bison)* (Todd e Storz 1983) and African buffalo *(Syncerus caffer)* (Rossiter et al. 1989; B. Dewals 2006), where all the species demonstrated to support both natural or experimental BoHV-4 infections. Interestingly, it has been observed that BoHV-4 infection can protect African buffalo from malignant catarrhal fever disease (Rossiter, Gumm, e Mirangi 1988). BoHV-4 has also been identified in non-ruminants species, like cats (*Felis catus*) (Fabricant, Gillespie, e Krook 1971; Kruger et al. 1990) and lions (*Panthera leo*) (L. Egyed, Kluge, e Bartha 1997; Kruger et al. 2000). The cat isolate has been initially, erroneously, designated as feline herpesvirus 2 (Saul Kit et al. 1986). Moreover, BoHV-4 infected cats represent a well distribuited populations across USA (Kruger et al. 2000). Rabbits, guinea pigs (L. Egyed, Kluge, e Bartha 1997), deer and owl monkeys (*Aotus trivirgatus*) from an American primate centre (Bublot et al. 1991), have also been found to be BoHV-4 infected animal species.

Despite BoHV-4 presence has been detected and demonstrated from many different tissues, its persistence site in both natural and experimental hosts are the monocyte-macrophage cellular lineages. Moreover, despite many γ-herpesviruses, such as Epstein-Barr Virus (EBV) and Herpesvirus Saimiri (HVS), showed growth-transforming capacity into persistently infected cells, there are no BoHV-4 growth-transformation evidences to date. In fact, there are no BoHV-4 genes homologs with transformation-associated genes of the other γ-herpesviruses. This feature represents a fundamental safety advantage in order to exploit BoHV-4 as an antigens delivery vector for therapeutic goals. (Gaetano Donofrio, Herath, et al. 2007)

Pathogenical role

BoHV-4 has been initially isolated from cattle affected by various diseases, such as malignant catarrhal fever, respiratory diseases, skin lesions and genital tract diseases, such as vulvovaginitis, abortion and post-partum metritis, but also from healty cattle (Zimmermann et al. 2001). Particularly, metritis represents a concerning bovine post-partum uterine disease affecting approximately 40% of cows(Gaetano Donofrio, Herath, et al. 2007). In 1973, Parks and Kendrick (Parks e Kendrick 1973) have been the first to document BoHV-4 isolation from a metritis affected cow and, to date, since it is always identified whit endometritis, abortions and mastitis cases, BoHV-4 could be considered the most common potential uterine viral pathogen. However, BoHV-4 serology or isolation is not usually

performed on metritis affected animals, although its endometrial cells tropism. *In vitro* experiments have demonstrated that BoHV-4 is efficiently able to infect purified endometrial epithelial and stromal cells and induce viral replication through non-apoptotic cellular death. Since BoHV-4 possesses the ability to enter many different animal species cell types, its endometrial tropism and successful replication must be related to post-entry events into host cells. BoHV-4 lytic switch is controlled by the Replication and transcription activator (Rta) Immediate Early 2 (IE2) gene, encoded by ORF50 and highly conserved among Rhadinoviruses (Gaetano Donofrio et al. 2008).

To date, BoHV-4 pathogenic role remains not clearly demonstrated and only in some cases it has been possible to experimentally reproduce the pathologic clinical signs (E. Thiry et al. 1989). Donofrio et al. (G. Donofrio et al. 2000; Gaetano Donofrio e van Santen 2001; Gaetano Donofrio et al. 2005) conduced several studies highlighting the monocyte-macrophage cellular lineage as BoHV-4 putative latency persistence site, both in persistently infected cattle then experimental hosts, and although BoHV-4 presence has often been associated with metritis onset (Frazier et al. 2002), they have developed an *in vitro* experimental system showing how BoHV-4 lytic cycle can be reactivated following a concurrently bacterial infection, thus demonstrating BoHV-4 potential secondary non-etiological role in metritis-affected cows. This suggested mechanism can also explain why BoHV-4 can also be detected from healthy cattle in absence of bacterial inflammation. It is well known, indeed, that, in addition to BoHV-4, also bacteria are ubiquitally associated to metritis, and among which, *Arcanobacterium pyogenes and Escherichia coli* (*E. coli*) represent the most commonly identified uterine bacterial pathogens. In metritis-affected and BoHV-4 persistently infected cows, bacterial infections recruit BoHV-4-latently infected macrophages from peripheral lymphatic sites through bloodstream to the inflammation site where, bacterial co-infection induce viral lytic cycle resume. Mobilization of BoHV-4 persistently infected macrophages under bacterial infection signals, represents the most plausible pathway through which BoHV-4 can reach the uterine inflamed site and actively replicate into endometrial stromal cells. Bacterial LPSs (Lipopolysaccharides) induce COX-2 (cyclooxygenase-2) expression and PGE-2 (prostaglandin-2) synthesis from bovine endometrial stromal cells, that rapidly trans-activate the BoHV-4 replication master switch IE2 gene promoter. In turn, released viral particles infecting the endometrium can establish a positive feedback loop between infected endometrial cells PGE2 production and viral replication (Gaetano Donofrio et al. 2008). Moreover, Donofrio et al. demonstrated that IE2

protein trans-activates IL-8 gene promoter of *in vitro* BoHV-4 infected primary BESCs (Bovine Endometrial Stromal Cells), suggesting that chemokine IL-8 secrection might play a virulence mechanism to attract more susceptible cells to the inflamed site and amplify BoHV-4 positive feedback (Gaetano Donofrio et al. 2010). This plausible network could also be involved into other damaged tissues experiencing viral-bacterial co-infection, and a better understanding of its molecular mechanisms might provide new useful terapeutic targets for infections treatments (Gaetano Donofrio et al. 2008). Since it is traditionally implied as an antiviral drug and it has shown to be protective against other different intracellular pathogens, INFγ has been proposed as a BoHV-4 lytic replication antagonist molecule. Donofrio et al. have observed that INFγ is produced by both uterine resident innate and adaptative immune systems cells and induces BoHV-4 IE2 gene down-regulation (Jacca et al. 2014).

Since BoHV-4 persistently infects the monocyte-macrophage cell lines, it can be flown through the blood stream to every part of the body, thus, despite its recognised association with genital diseases, if BoHV-4 does contribute to other pathologies it is not known. In 1989, when it was isolated from Indian cattle affected by ethmoidal tumors, BoHV-4 has been designated as a "passenger virus" (Moreno-Lopez et al. 1989). During primary infections and viral reactivation from latency, BoHV-4 could potentially be isolated from every bovine organs, thus making it hard to understand if the virus might play a pathogenic role to the lesion. BoHV-4 can be regularly identified from healthy cattle spleen, lung and liver homogenates (Krogman e McAdaragh 1982), kidney cell cultures (Luther, Bradley, e Haig 1971; Belak e Palfi 1974) and trigeminal ganglia (Homan e Easterday 1981).

The European Movar 33/63 (Bartha, Juhász, e Liebermann 1966) and the American DN 599 (Mohanty, Hammond, e Lillie 1971) strains have been isolated from cattle with ocular and respiratory disease clinical signs, while several African BoHV-4-like strains, even if not yet recognised as BoHV-4, have been isolated from cases of vaginitis, epididymitis (Mare 1961; Theodoridis 1978; 1985) and from sperm. (Loretu et al. 1974) The Belgian LVR40 and V-Test strains have been isolated from cases of post-partum metritis and orchitis, respectively (Wellemans et al., 1984; Thiry et al., 1981). In Italy, BoHV-4 strains have been isolated from cases of genital diseases (Castrucci et al. 1986) and other strains from aborted fetuses in USA (De, Tj, e Me 1979) (Reed et al., 1979; Wellemans and van Opdenbosch, 1989). Moreover, Donofrio et al. have isolated an Apathogenic BoHV-4 (BoHV-4-A) strain from the milk cellular fraction of persistently infected healthy cows (G. Donofrio et al. 2000).

To date, therefore, BoHV-4 infection is believed to occur as sublinic form in most cases even if its presence is always suspected to be associated with post-partum metritis and abortion cases, while it is doubtful wheather if it is involved into ocular and respiratory diseases (Izumi et al. 2006).

Detection. BoHV-4 can be easily detected from vaginal and nasal secretions but also from both primary and immortalized bovine cell lynes obtained from organs homogenates. Bovine Embryo Kidney (BEK), Madin Darby Bovine Kidney (MDBK), but also bovine, goat, sheep, cat, rabbit, pig and chicken primary cell lines are usually used for BoHV-4 culture, and since its replication is associated with the synthetic (S) cellular phase, cell cultures must be sub-confluently cultured in order to optimize BoHV-4 isolation (Vanderplasschen et al. 1995). Specific PCR (Polymerase Chain Reaction), detecting glycoprotein B (gB) (Boerner et al. 1999) and thymidine kinase (TK) (L. Egyed et al. 1996; L. Egyed e Bartha 1998; Wellenberg et al. 2001) genes, can be used to identify BoHV-4 DNA (L. Egyed et al. 1996; Boerner et al. 1999), whose identification would just reflect the presence of productively or latently infected mononuclear blood cells, but it would not tell whether BoHV-4 is responsible for the lesion.

Since BoHV-4 induce very low or not detectable levels of neutralizing antibodies titres in infected cows, serum-neutralization assay is not usually performed for virus identification. The weak host humoral immune response might be due to BoHV-4 capacity to minimally expose its antigens to the antibodies of the immune system. Machelis et al. (Machiels et al. 2011) have observed that BoHV-4 envelope glycoprotein gp180 is responsible for gB, gH-gL epitopes protection from antibodies neutralization. They have demonstrated that serum neutralization can be very effective on recombinant gp180-deficient BoHV-4 immunised animals. Indirect Immunofluorescence test (IFAT), ELISA assay and Immunoperoxidase Monolayer Assay (IPMA) are alternative techniques that have been standardized to detect BoHV-4 seroconversion 14-20 days post primary infection (Dubuison et al. 1987; Osorio e Reed 1983).

To date, no vaccines against BoHV-4 are present in Europe and its control and prevention is exclusively achieved through hygienic measures (Etienne Thiry et al. 2000).

Genome structure

In 2001, Zimmerman et al. genome sequencing and molecular data allowed BoHV-4 classification as a gammaherpesvirus (Zimmermann et al. 2001). BoHV-4 genome is a B-like linear double-stranded DNA of 144±6 Kbp, consisting of a ±108 Kbp, GC poor, central Long Unique Region (LUR) and approximately fifteen 200 bps, GC rich, non-coding tandem repeats, designated as polyrepetitive DNA (prDNA), located at both ends (Ehlers, Buhk, e Ludwig 1985). prDNA sequences are also designated as Heavy DNA (H-DNA) or terminal tandem repeats. 58% of BoHV-4 overall genome is organized into five, 5'-3'-oriented, genes blocks, numbered from 1 to 5 (László Egyed 2000). Specifically, blocks 1, 2 and 4 contain several genes well conserved among α-, β- and γ-herpesviruses, while specific γ-herpesviral genes are contained into blocks 3 and 5 (*Fig.1*). Moreover, it has been observed that lenght and genetic content of the genome intergenic regions differ among herpesviruses (Markine-Goriaynoff et al. 2003; Morán et al. 2015a). The bovine herpesvirus-4 (BoHV-4) group comprises a collection of antigenically related viruses, all presenting the same schematic genome organization, distinct from the other bovine herpesviruses (E. Thiry et al. 1989). However, BoHV-4s of this group are further distinguished from each other into three categories, based on three main differences, one occurring in prDNA unique region and the other two in the LUR. HindIII, EcoRI and BamHI restriction maps as well as lambda and plasmid libraries analysis, allowed the identification of the DN-599, MOVAR 33/63 strains-like categories and a third "not classified" one (Markine-Goriaynoff et al. 2003).

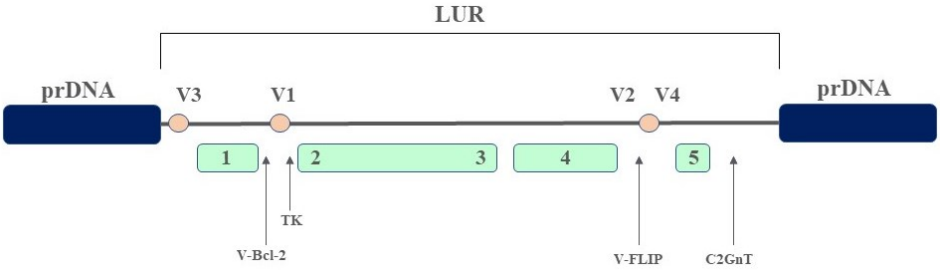

Figure 1. **BoHV-4 genome schematic organization.**

Legend: prDNA= polyrepetitive DNA; LUR= Long Unique Region; V1-V4= variable sequences; 1-3= conserved genes; V-Bcl-2, V-FLIP= anti-apoptotic genes; TK= Thymidine Kinase; C2Gnt= β-1,3-1,6-N-acetylglucosaminyltransferase.

prDNA

Genome analysis of BoHV-4 66-p-347 strain revealed that prDNA complete nucleotide sequence contains 2267 bp and an average 70% of G-C content. The prDNAs number varies at each end of the genome, however, the overall copies number remains approximately constant, of about 15 prDNA units for each strain (Ehlers, Buhk, e Ludwig 1985).

Every prDNA unit contains two different repeated sequences, one at the 3' terminus of about 196-197 bp, characterized by the presence of three complete and one incomplete direct repeats, and a second one, twice repeated, at the 5' terminus of 25 bp. Shorter repeats are separated by AGA/GGC triplette (H. Broll et al. 1999; Markine-Goriaynoff et al. 2003).

prDNA units sizes are generally strain-specific, but small fractions may vary within each strain (Bublot et al. 1990). Eight different prDNA sizes, from 1450 to 2850 bp, have been observed. These sizes differences seem to be related to the presence of a variable number of 200 bp fragments called "hot spots", inside the prDNA unit, that are probably involved in recombination events (Morán et al. 2015b). This fact should not be surprising since G-C rich tandem repeats in eukaryotic genomes are well known to be involved in these kind of processes (Bublot et al. 1990).

Chang & Van Santen did not detect any RNA transcripts from neither one of the two prDNA sequences. However, based on LUR ends mRNAs similar sizes, they suggested that RNA transcription proceed over genome fused ends and that prDNA direct repeats sequences are removed during the RNA processing (L.-Y. Chang e Van Santen 1992).

Different studies demonstrated that these polyrepetitive sequences are involved into genome excision and packaging processes, thus defining mature virions genome size (Morán et al. 2015b; González Altamiranda et al. 2015). It has also been observed that one prDNA is already enough for cleavage and packaging processes.

It has, indeed, been *in vitro* observed that BoHV-4 genome repeted termini get fused together during the viral lytic cycle in order to allow DNA rolling circle replication (Zimmermann et al. 2001). prDNA units sequencing clarified that herpesviral genome cleavage and packaging processes take place at two conserved cis-acting signals, designated as *pac-1* and *pac-2*, and respectively located at BoHV-4 genome left and right ends (*Fig.2*). *Pac-1* C and G stretches and *pac-2* 23-33 nt T motif and C-G rich region are locally defined and well conserved among α-, β- and γ-herpesviruses, even if herpesviruses share low *pac1* sites overall homology. As previously described for bacteriophages (Black 1989; Davison 1992; Baines et al. 1994), herpesvirus cleavage and assembly mechanisms could be mediated by a virus encoded terminase cleavage at a precise and defined distance from *pac-1* and *pac-2* sites of

the circularized concatemeric genomes, generating single DNA monomeric units into preassembled capsids (H. Broll et al. 1999; Hermann Broll et al., s.d.). Broll et al. demonstrated for the first time that a 443 bp prDNA is sufficient to guarantee cleavage and packaging of a herpesviral B-type genome (H. Broll et al. 1999), thus confirming Ehlers et al. hypothesis of prDNA playing a "genome-size buffer" role, ensuring the packaging of a complete genome copy, LUR and prDNAs included, thus playing a key role in BoHV-4 replication cycle (Ehlers, Buhk, e Ludwig 1985). Previous studies revealed that also Herpes Simplex Virus type 1 (HSV-1) possesses the same signal sequences involved into viral DNA cleavage and packaging (Deiss, Chou, e Frenkel 1986; H. Broll et al. 1999). At least seven genes are involved into HSV-1 genome cleavage-assembly (Osterrieder 2017); among these genes, the highly conserved UL15 gene encodes for the viral terminase catalytic subunit. Evident aminoacidic sequence homologies between HSV-1 and BoHV-4 proteins showed that HSV-1 UL15 terminase related protein is similar to BoHV-4 ORF29 encoded protein, thus playing the key catalytic role during γ-herpesviral DNA cleavage (Hermann Broll et al., s.d.). Subsequently, *pac-1* and *pac-2* sequences have been discovered at the genomic termini of all herpesviruses (Deiss, Chou, e Frenkel 1986; H. Broll et al. 1999). In addition of playing a key role in rolling cicle viral replication, prDNAs seem to be involved into other functions. Collins et al. demonstrated for the first time that intact and multiple prDNAs are essential for genome episomial structure manteinence into γ2-herpesviruses latently infected T cells. prDNAs cis-acting signals seem to interact to viral Latency-Associated Nuclear Antigens (LANA), promoting episome attachment to the mitotic chromosomes thus assuring the episomal state maintenance and contemporaly its segregation to progeny cells (Cotter e Robertson 1999; Collins et al. 2002).

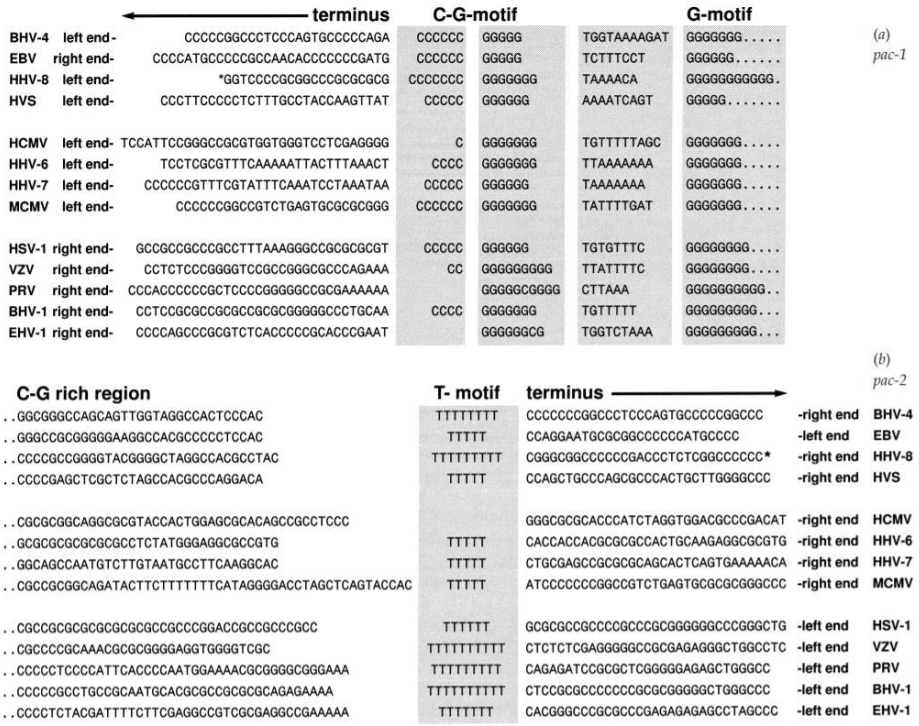

Figure 2. **Schematic representation of BoHV-4 *pac-1* and *pac-2* conserved sequences at genomic termini.** Adapted from (H. Broll et al. 1999).

5'-3' (left to right) alignment of BoHV-4 *pac-1* (a) and *pac-2* (b) motifs among 12 α-, β- and γ-herpesviruses (α-herpesviruses: HSV-1 = Herpes Simplex virus type 1; VZV = Varicella Zoster virus; PRV = Pseudorabies virus; BoHV-1 = Bovine herpesvirus type 1; EHV-1 = Equine herpesvirus type 1. β-herpesviruses: HHV-6/7 = Human Herpesvirus type 6/7; HCMV = Human Cytomegalovirus; MCMV = Murine Cytomegalovirus. γ-herpesviruses: EBV = Epstein Barr virus; HHV-8 = Kaposi's sarcoma associated herpesvirus; HVS = Herpesvirus Saimiri). Pac1 and pac2 conserved signals are enclosed in grey boxes, specifically aligned for each herpesvirus.

LUR

Shotgun sequencing of cloned herpesviral DNA fragments determined a 108,873 bp LUR consensus sequence with 41.4% of C-G content. As all the other γ-herpesviruses, BoHV-4 LUR sequence contains very low CpG dinucleotides frequency, of about 1% for BoHV-4, compensated by an excess of CpA and TpG content. Herpesviruses lack, indeed, of DNA methilation systems, which are higly exploited from higher eukariyotes to control their genes expression; thus, BoHV-4 low CpG content might be related to its latency state during cell

division, in order to force its switch to replication phase during cell mytosis (Honess et al. 1989; Markine-Goriaynoff et al. 2003).

Within γ-herpesviruses LUR, two multiple direct repeats regions, designated R1 and R2, have been detected. R1 contains 23, 25 and 65 bp complete and incomplete direct repeats stretches, located at 21858-22631 bp position. R1 repeats number, lenght and sequence vary among different BoHV-4 strains (Bermudez-Cruz, Zhang, e van Santen 1997). R2 contains two different repeats stretches, designated as R2a and R2b. Also in this case, R2 lenght, location and sequence are specific for each BoHV-4 strain. R2a consists of 28 perfect and 3 imperfect direct repeats of 22-23 bp, located at 96250-97034 bp LUR position. Some R2a direct repeats are interspaced by 2-3 GGAAG motifs, 15 in total. R2b, instead, contains several different 8-68 bp direct repeats and one inverted repeat, located at 97565-97616 bp position, with a RNA hairpin loop predicted structure. Compared to the overall LUR sequence, R2a shows a high C-G content, of 71%, while a lower 30% is located into the 750 bp stretch upstream R2. A further extensive analysis of a 60Kbp genome stretch, comprising 45% of LUR and five prDNAs units, allowed the identification and mapping of BoHV-4 origin of replication (ori), sharing positional omology with the lytic origin of replication (*oriLyt*) of other gamma herpesviruses, like EBV. BoHV-4 ori is localized, downstream ORF69, into R2b stretch, closely positioned to a C-G rich region. BoHV-4 Ori partially overlaps with Bo11 gene and include Bo12 ORF sequence, even if these genes envolvment into viral DNA replication has yet to be clarified.

Zimmermann et al. have identified 79 Oper Reading Frames (ORFs) in BoHV-4 LUR 66-p-347 strain (*Fig.3*), based on three research cryteria: 1- start and stop codons presence; 2- 180 bp as minimal size; 3- less than 50% overlapping with other ORFs. 17 of the 79 ORFs specifically belong to BoHV-4, designated as “Bo” and followed by a number indicating the positional order within the LUR sequence (5’-3’: Bo1-Bo17), while the other 62 ORFs share homologies with HHV-8 and HVS genes, mantaining their nomenclature (Zimmermann et al. 2001). These conserved genes are all organized in common blocks among γ-herpesviruses genomes but differ for orientation, number and position (Neipel, Albrecht, e Fleckenstein 1998; Simas e Efstathiou 1998).

Specifical differences have been found for ORF16 (also designated as viral B-cell lymphoma (v-Bcl-2)) position and presence/absence, and for ORF50 and 69 individual or subgroup-specific genes number.

BoHV-4 ORF50 encodes for Immediate Early 2 (IE2) protein, highly conserved among Rhadinoviruses, playing a key role in γ-herpesviruses lytic cycle induction (Gaetano Donofrio, Cavirani, et al. 2004).

BoHV-4 genes arrangement is similar to HVS, as they both miss long non-coding stretches within LUR sequence and posses 16-69 equally oriented ORFs stretch into LUR central part. Along this stretch, Bo9 and Bo10 genes are genetically unique of BoHV-4 but share the same position and orientation with HSV ORFs. However, HVS, as well as other γ-herpesviruses with declared transforming capacity, posses several ORFs (1, 2, 4, 5, 11-15, 28, 51, 70, 72 and 74), sharing homologies with cellular genes, involved into cellular growth and survival, nucleotides metabolism and immune escape, such as cytokines-, cytokines and iterleukins receptors-, chemokines- or viral macrophage inflammatory protein α/β-genes, that have no homology at all with BoHV-4. These cellular homologues genes have probably been acquired from cellular host genomes in the past, and might played a key role during herpesviruses evolution. To date, even if it has never been officialy demonstrated, BoHV-4 is not related neither to lympho-proliferative disorders or transforming ability, which, instead, are generally attribuited to other γ-herpesviruses, such as HHV-8 or MuHV-68. However, BoHV-4 ownes ORF16 and ORF71 encoding for viral B cell lymphoma (v-Bcl-2)-like and viral FLICE Inhibitory Protein (v-FLIP) repectively, involved into host cell cycle regulation. It is known that v-Bcl-2 and v-FLIP play a potential role in the early stage of cellular transformation, preventing apoptosis. Many viruses possess genes encoding for apoptosis inhibitor proteins and exploit them to establish a persistent infection or to prolong their survival into infected cells, thus maximizing their viral production. BoHV-4 BORFE2 protein is able to inhibits FLICE Associated Signal (Fas)- and Tumor Necrosis Factor Receptor 1 (TNFR1)-induced apoptosis; moreover, like other γ-herpesviruses, it contains two Death Effector Domains (DEDs). Fas is involved into cytotoxic T cell killing of virus-infected cells, while TNF can directly kill them. Even if the exact ihibition mechanisms are not completely understood, two different potential mechanisms have been hypothetized. One suggesting BORFE2 interaction with the caspase 8 pro-domain, thus inhibiting its binding to Fas-Associated protein with Death Domain (FADD) and preventing caspase activation. A second one suggesting that BORFE2 interaction to the caspase 8 pro-domain might modify its conformation and prevent its activation (G. H. Wang et al. 1997).

Even if BoHV-4 has been shown to harbor genes with antiapoptotic potentialities, Bellows et al. (Bellows et al. 2000) experiments have observed that herpesviruses v-Bcl-2 tend to lose anti-apoptotic activities by escaping caspase-mediated conversion into pro-apoptotic

proteins. However, by using different techniques for apoptosis detection and using different cell targets and virus strains, Sciortino et al. demonstrated that, despite the presence of anti-apoptotic genes in its genome, BoHV-4 productive infection is associated with apoptosis instead with a lytic phase. This feature is also shared by other gamma2-herpesviruses (Sciortino et al. 2000).

BoHV-4 ORF21 is a 1335 nt long sequence, involved into nucleotides metabolism, encoding for Thymidine kinase (TK), necessary for pyrimidine metabolism and nucleic acids synthesis.

Viral genes capable of increasing nucleotides amount into host-infected cells affect not only viral replication but also host cell proliferation itself (Neipel, Albrecht, e Fleckenstein 1998). Zhang et al. (L. Zhang e van Santen 1995) demonstrated that BoHV-4 TK gene promoter regulatory region is specifically trans-activated by IE2 protein.

Also ORF3 and 75, located in opposite directions at LUR ends, encoding for viral phosphor ribosyl formyl glycinamidine synthetase (v-FGAM), increase the nucleotides amount in infected cells, favoring both viral replication and cell proliferation (Zimmermann et al. 2001).

BoHV-4 unique Bo17 gene, encoding for β-1,6-N-acetylglucosaminyltransferase (β-1,6GnT/C2Gnt), is actively involved in viral replication, particularly in mononuclear blood cells replication, and no other known viruses posses it (Vanderplasschen et al. 2000). It is located at LUR right end and its product shares 81.1% similarity with the human β-1,6GnT homologous (Morán et al. 2015b). β-1,6GnT genes family are known to encode for enzymes involved in glycans synthesis but also in pathological processes, like oncogenesis and immunodeficiency, when mutations events occur (Vanderplasschen et al. 2000); thus, based on this fact, under certain conditions, BoHV-4 could potentially trigger lymphoproliferative or transforming events. Interestingly, phylogenetic analysis of 34 different BoHV-4 strains derived from different animal species around the world, highlighted that Bo17 seems to represent BoHV-4 most recently acquired gene. Specifically, it is 1.5 million years ago dated and seems to derive from the Syncerus lineage rather than the Bos one. The transition to a cattle ancestor may then have occurred through an African buffalo ancestor, about 700,000 years ago (Markine-Goriaynoff et al. 2003).

Lété et al. observed that, differently from the cellular one, BoHV-4 β-1,6GnT encodes for two different mRNAs, one corresponding to the entire gene coding region, and a second one deriving from splicing of a 138 bp intron encoding for critical enzyme residues. Enzymatic

assays did not show any particular activities related to the spliced mRNA form or replication differences between the two mRNA types (Lété et al. 2016).

BoHV-4 expresses Bo17 during the early replication stage and it has been observed to be indispensable for *in vitro* viral productive infection. However its contribute to *in vivo* BoHV-4 biology is still not clear. An hypothesis suggest its *in vivo* contribute to viral proteins post-translational modifications by the addition of glycans, thus influenzing viral tropism or its susceptibility to antibodies neutralization, cell-mediated cytotoxicity or complement lysis, in order to favor viral escape from the host immune system defenses (Markine-Goriaynoff, Gillet, Karlsen, et al. 2004).

This hypothesis might be highly plausible since viruses have constantly co-evolved with their hosts by developing different strategies in order to manipulate host immune mechanisms to favor their life cycle (Markine-Goriaynoff, Gillet, Van Etten, et al. 2004; Vigerust e Shepherd 2007).

BoHV-4 Bo1-Bo17 unique ORFs, except for Bo5, have no homologies with other herpesviruses. Among these, Bo1, 3, 6, 7, 12, 13, and 15, have not yet been described.

Bo4 and spliced Bo5 ORFs encode for part of the major Immediate-Early 1 (IE1) trascript, as previously described by van Santen (Bermudez-Cruz, Zhang, e van Santen 1997), and both genes seem to be involved in differential splicing.

In addition to Bo4 and 5, other genes, like Bo10, Bo11, ORF29, ORF50 and ORF57 undergo differential splicing processes. Bo5 ORF also shares 27,85% aminoacidic homology with KSHV K5 ORF (Haque et al. 2000).

ORF29 product is a terminase involved into viral DNA cleavage and encapsidation processes (H. Broll et al. 1999).

ORF50 has been shown to be encoded by the spliced IE2 gene, and its product is a putative R transactivator (van Santen 1993).

ORF57 product seems to play a key role in viral mRNAs processing, as it seems to be involved into spliceosome complex redistribution of HVS infected cells (Cooper et al. 1999).

Bo8 ORF partially overlaps the 1.7Kbp late RNA described in 1997 by Bermudez-Cruz et al (Bermudez-Cruz, Zhang, e van Santen 1997).

Bo10 ORF is positionally homologous to KSHV K8.1 and EBV gp350 genes, and encodes for the late 180 KDa gb180 protein during viral replication. gp180 is a non-essential BoHV-4 envelope protein involved into Glycosaminoglycans (GAGs) dependent viral attachment. Machiels et al. observed that gp180 deficient virions show growth deficit, being slower than Wild Type (WT) ones to GAG^+ cells attachment, but showing enhanced infectivity for GAG^-

cells. Based on MuHV-4 gp150 analogies, it seems that BoHV-4 gp180 allow GAG dependent cell attachment by partially hiding other BoHV-4 GAG independent viral attachment epitopes (BoHV-4 gB, gH and gL glycoproteins complex). Bo10 deleted virions, however, do not show faster or easier cell binding, thus gp180 might protect other virion entry proteins more suscettible to antibodies neutralization, avoiding a seroneutralizing host response (Machiels et al. 2011).

Lastly, Bo11, even if it doesn't satisfy Zimmermann rule, being shorter than 180bp ORF lenght, is encoded by an Immediate Early 2 (IE2) transactivated, spliced 1.1Kbp late RNA, previously described by Bermudez-Cruz et al. (Bermudez-Cruz, Zhang, e van Santen 1997).

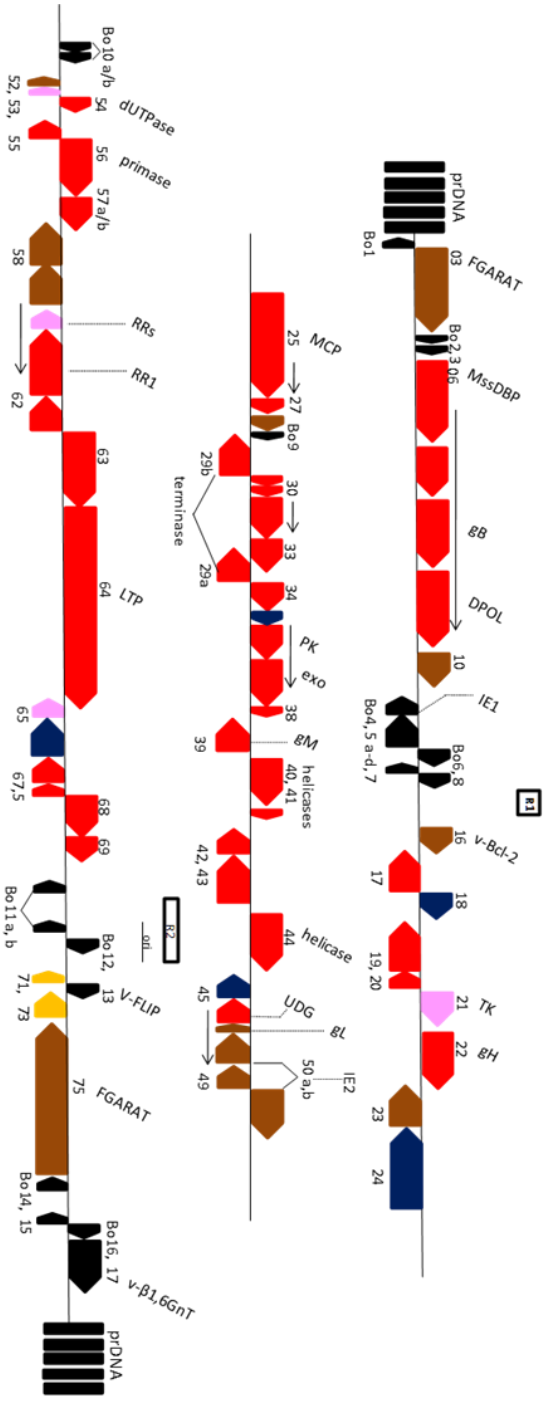

Figure 3. **Schematic representation (not on scale) of BoHV-4 genome map.** Adapted from (Zimmermann et al. 2001)

- LUR is represented as a line
- ORFs are represented as arrows with the right orientation
- Homologous ORFs to other gammaherpesviruses are coloured in red while BoHV-4 unique ORFs are coloured in black

Interblock regions

Interbloks regions represent the herpesviral genome most variable part, where virus-specific genes, involved into several biological viral functions, are located (Lomonte et al. 1996). The BoHV-4 five interblock regions, named A-F, have been deeply analyzed and sequenced in orded to identify its peculiar genes. Each region count an amount of 23000 nt with an average of 43% G-C content (Bublot et al. 1990; László Egyed 2000).

Twelve potential ORFs and several non-coding regions have been identified among all the interblocks regions. To highlight that these are BoHV-4 specific ORFs, each one has been named with the BORF- prefix, followed by the interblock region letter and the positioning numer if more thatn one ORF belong to the same interblock. Two BORFs, BORFA1, BORFA2 and BORFB1, BORFB2 belong to to regions A and B respectively, while no ORF have been detected for region C. BORFD1 only, belongs to region D, while three BORFE1-3 and four BORFF1-4 respectively, belong to region E and F. Based on position and orientation similarities, HVS ORF3,10,16,51,71 and 73 seem to be homologous to BoHV-4 BORFA1, BORFB1-2, BORFD1, BORFE2 and BORFF4, while no similarities with other herpesviruses have been observed for the other BORFs, unique of BOHV-4 (Lomonte et al. 1995). This additional similarity evident with HSV, further corroborate BoHV-4 nature of being a gamma herpesvirus. To date the only established difference between the two viruses is BoHV-4 cellular homologous genes absence (Lomonte et al. 1996).

Viral structure

BoHV-4 viral particles measure overall 200nm in diameter and are superficially enclosed in a lipid envelope, studded with glycoproteins involved in adhesion and penetration during the early viral infectious stages. They internally consist of an icosahedral capsid composed of 12 pentamers and 150 hexamers, surrounded by a tegument layer of about 15 different proteins whose organization and function have not yet been clarified. The tegument layer is acquired both in the cytoplasm and in the nucleous during viral replication. Between the tegument and the external envelope there is a 100nm in diameter large nucleocapsid which is generated and assembled into the host cell nucleus by at least 8 different proteins (*Fig.4*) (Lété, Palmeira, et al. 2012).

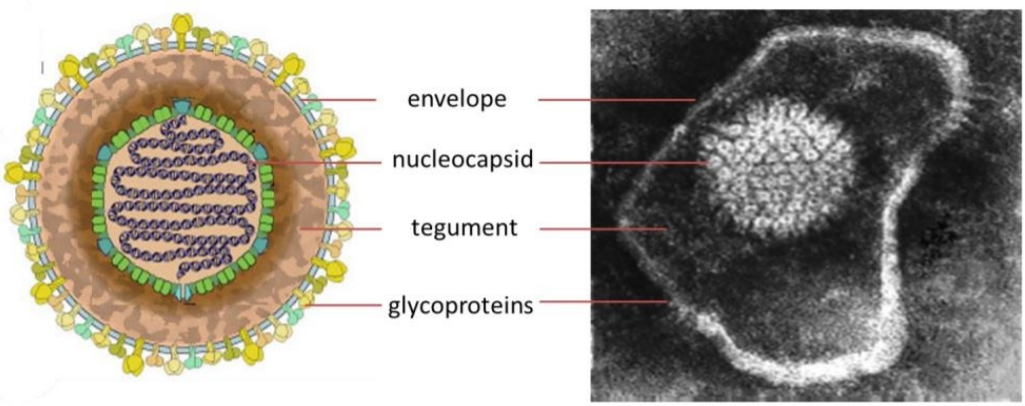

Figure 4. **BoHV-4 structure.** Adapted from (Riaz, Murtaz-ul-Hasan, e Akhtar 2017).
On the left: a typical herpesvirus virion schematic representation, showing the major structural components. On the right: HSV electron micrograph, showing the same respective major structural components

All different proteins composing viral particles do not simply define their structures but also actively support their life functions, maximizing their replication potential. Particularly, envelope glycoproteins have been shown to mediate several viral replication steps, such as attachment, entry, egress and virions spread, and to influence viral interactions with the host immune system, as well as with infected cells (D. C. Johnson e Spear 1982).

To date, Lété et al. analysis of BoHV-4 protein profile identified five envelope glycoproteins and several major glycoprotein complexes. The first to be discovered was the gp6/gp10/gp17 complex, representing BoHV-4 gB envelope glycoprotein (Lété, Palmeira, et al. 2012). Following the gp11/VP24 complex, of 120/16.5 KDa, where VP24 polypeptide is non-covalently linked to the N-glycosylated gp11 (Dubuisson et al. 1992). A third 135 KDa gp8 envelope major glycoprotein, involved in BoHV-4 attachment to host cell surface (Vanderplasschen et al. 1993), and a fourth gp1 glycoprotein of over 300 KDa, that have been proved to be N-glycosylated after tunicamycin and glycosidases treatments (Dubuisson, Pastoret, e Thiry 1992) have been discovered. In addition, another 26/27 KDa gp21 glycoprotein has been detected in infected animal sera (Dubuisson, Thiry, Bublot, Thomas, et al. 1989) and other two gp110 and gp31-35 glycoproteins, homologues to other herpesviruses gH and gL envelope proteins, have been identified (Lété, Palmeira, et al. 2012).

Envelope proteins. B, H, L, M and N glycoproteins (gB, gH, gL, gM and gN) are five envelope glycoproteins well conserved among herpesviruses (Dubuisson, Thiry, Bublot, Sneyers, et al. 1989; Franceschi et al. 2013). Lété et al. detected five BoHV-4 envelope proteins, gB, gH, gL, gM and gp180, among which gB, gH and gp180 are the most glycosylated. Moreover, they predicted other four ORF27-, ORF53-, ORF58- and Bo9-encoded potential envelope proteins into BoHV-4 genome. No peptides corresponding to gN glycoprotein have been detected. BoHV-4 gM detection but gN absence was unexpected since in all herpesviruses gN forms a complex together with gM and is essential for its proper processing, thus it has been hypothesised that gN could dissociate from gM during mature virions budding, explaining its absence from mass spectrometry analysis.

Interestingly, Romeo et al. recently demonstrated that differences of envelope glycoproteins time expression and post-translational glycosilation protect BoHV-4 from seroneutralization, thus explaining animals low or absent neutralizing antibodies production after BoHV-4 infection (Romeo et al. 2020).

gB. BoHV-4 gB is a 873aa peptide encoded by the 2622bp long ORF8 and is responsible of binding the host cell heparan sulfates during viral attachment. Heparan sulphate proteoglycans represent the predominant GAG molecules involved into the attachment stage of herpesviruses lifecycle.

BoHV-4 gB mature and active structure is represented by gp6-gp10-gp17 heterodimer of 150, 120 and 51KDa respectively, representing the first discovered BoHV-4 major complex. gp10-gp17 derives from the proteolytic cleavage of a 135 kDa translation product of the BoHV-4 gB gene and the two subunits are held together by disulfide bonds. On the contrary, gp6 is non-covalently linked to the complex. Deeper analysis showed that gB N terminus is highly glycosilated, presenting fourty O-glycosylation sites (Lété, Palmeira, et al. 2012).

gB homologues are widely conserved among *Herpesviridae* but whether its interaction is sufficient for BoHV-4 adsorbition or if specific cellular receptors are required for its attachment is not yet well understood (Lété, Palmeira, et al. 2012). Interestingly, Franceschi et al. *in vitro* generated a recombinant gB-deficient BoHV-4, BoHV-4-A-ΔgB, in order to demonstrate that B glycoprotein is indispensable for BoHV-4 replication as its deletion impedes its infectious capacity and survival (Franceschi et al. 2013).

gH; gL. Herpesviruses cell entry is mediated by host cell membranes and viral envelopes fusion. Based on virus and target cell types, herpesviruses host cell entry could be mediated by direct cell membrane-viral envelope fusion as well as pH-dependent/independent endocytotis. Moreover different herpesviruses can exploit different multiple host cell

receptors, thus complicating herpesvirus fusion mechanism understanding. BoHV-4 gH and gL interact to each other forming the gH-gL heterodimer, that, together with gB represent a core fusion machinery, which is well conserved among all three herpesvirus subfamilies. Although gL has been proven to be indispensable for alpha and beta herpesviruses entry by regulating gH correct folding and incorporation, it is not essential for BoHV-4 infectivity. Lètè et al. generated a gL deficient BoHV-4, BoHV-4-gL STOP, by introducing stop codons into ORF47 signal peptide sequence, and demonstrated that gL lack does not inhibit infectivity but reduce viral growth. This is beacause BoHV-4 gL seems to drive endocytosis rather than gH regulation and so direct membranes fusion. Its lack compromises viral entry but it does not entirely prevent it (Lété, Machiels, et al. 2012).

gM. BoHV-4 gM function has not yet been described. As previously described gM seems to form a complex together with gN, necessary for its proper processing (Lété, Palmeira, et al. 2012).

gp180. BoHV-4 gp180, as previously described, is encoded by Bo10 gene and it has been demonstrated to be a structural protein, not essential for *in vitro* viral replication (Machiels et al. 2011).

Capsid proteins structure and arrangement are well conserved among *Herpesviridae* but designated with different names.

Herpesviruses possess a T=16 icosahedral capsid consisting of 12 pentamers made of five Major Capsid Protein (MCP), each penton representing one of the twelve icosahedral vertices, and 150 hexamers each made of six MCP. Pentamers and hexamers are linked together by 320 triplexes units, each composed of two ORF26- and one ORF62-encoded proteins. Triplexes are associated to ORF19 and ORF32-encoded capsid proteins forming the Capsid Vertex Specific Complexes (CVSCs), involved into DNA packaging and cleavage. An ORF65-encoded small protein interacts with MCPs all over the capsid shell surface. It has also been observed that ORF17.5-encoded scaffold protein and ORF17-encoded protease, that occupy virions capsid internal space, get displaced by viral genome during the DNA packaging stage of viral replication (Lété, Palmeira, et al. 2012).

Tegument proteins are indispensable for virions entry, assembly and egression. Comparison between Gammaherpesvirinae predicted the presence of about 15 proteins into BoHV-4 tegument, among which thirteen have been identified: ORFs 3, 33, 35, 38, 42, 45, 48, 52, 55, 63, 64, 67, 75-encoded proteins. However, the TK protein, which is a higly expressed

tegument component among gammaherpesvirus, has not been detected into BoHV-4 virions tegument. Similarly, the BoHV-4 ORF23-encoded protein, which is involved into capsid transportation through the trans Golgi network during virion egression, has not been detected into BoHV-4 tegument.

Lété et al. identified ORF52-encoded product as a BoHV-4 major tegument component, well conserved among γ-herpesviruses, but without homologues to both α-herpesviruses and β-herpesviruses. Based on Muride gammaherpesvirus 4 (MuHV-a) studies, it was discovered that ORF52 encode for a circa 20KDa protein, necessary during γ-herpesviruses tegumentation and secondary envelopment assembling (Lété, Palmeira, et al. 2012).

Life cycle

During herpesviruses *in vitro* replication, viral proteins expression is temporally regulated and, based on their synthesis chronological order, they have been classified into: Immediate-Early (IE or α), Early (E or β) and Late (L or γ) proteins. All three protein classes expression are finely controlled and regulated through a feedback mechanism (*Fig.5*).

IE proteins are the first to be synthetised right after viral genome release from the capsid into host cell nucleous, and act as control proteins for E and L genes expression but also for their own expression. Moreover, IE proteins induce cellular DNA synthesis and cell cycle arrest, favoring viral replication. The subsequently expressed E and then L genes, encode for enzymes (DNA polymerase, thymidine kinase, helicase and several transcription factors responsible for DNA replication) and structural capsid, tegument and envelope proteins, respectively. E proteins have also been observed to be involved in IE genes down regulation, and together with IE proteins to regulate L genes transcription.

L genes expression begins after viral DNA synthesis inside the host cytoplasm and most of the encoded structural proteins migrate back to the host nucleus, assembling to each others, to generate new virus capsids (Vanderplasschen et al. 1995). L genes can be distinguished into Partial Late (L1) and Real Late (L2) classes, based on their different temporal expression. L1 genes are earlier transcribed during viral infection and their expression is barely influenced by the presence of DNA synthesis inhibitor proteins, while L2 genes are later transcribed and their production is totally inhibited by the presence of DNA synthesis inhibitors. (Dubuisson, Pastoret, e Thiry 1991).

Particularly, Vanderplasschen et al. (Vanderplasschen et al. 1995) *in vitro* demonstrated that succesfull BoHV-4 replication is restricted to the cell cycle Synthesis (S) phase. Regardless the host cell type and the viral Molteplicity Of Infection (MOI), BoHV-4 genome and subsequent L proteins synthesis depends on cells S phase. Plaques generation kinetic is evidently faster on actively replicating freshly seeded cells compared to confluent cells monolayers, appearing 5-6 and 10-12 days post-infection, respectively. Contrary to what is usually observed for herpesviruses, BoHV-4 does not produce Virion Host Shutoff (VHS) proteins, probably in order to facilitate the cell replicative mechanism to reach S phase. Moreover, they also observed that BoHV-4 can block its replication at a late stage of infection, probably after E proteins expression and before L proteins synthesis, giving rise to a latent infection.
In addition, Drayman et al. (Drayman et al. 2017) demonstrated that, despite viral replication success mainly depend on cellular S phase, other factors, such as cell replication kinetic and cellular density, can strongly influence viral genes expression.

Herpesviruses are able to establish either a lytic than a latent infection. During latency, viral genome assume an episomal circular form, expressing only few viral genes, but cellular stress events, immunosuppression, tissue damages or UV rays or can resume viral replication from latency (Riaz, Murtaz-ul-Hasan, e Akhtar 2017). α- and β-herpesviruses usually establish lytic infections with viral replication and virus progeny release, while only a smaller cellular subset allows their latency. γ-herpesviruses, insted, seems to initially predilige the latency establishment. They have evolved to *in vivo* establish a lifelong persistent infection accurately balanced with the host immune system.
During latency, only a restricted amount of cells undergo latent infection while the host immune system, instead of destroying them, allow virus excretion and its transmission whitout showing disease symptoms. This balancing is mantained by infected cells low numbers and virus excretion low levels. However, in targeted immune deficient species and hosts whose immune system do not balance with herpesviral persistence, the amount of latently infected cells takes over the host immune control, developing lethal diseases, like Kaposi's sarcoma or malignant catarrhal fever (Ackermann 2006).

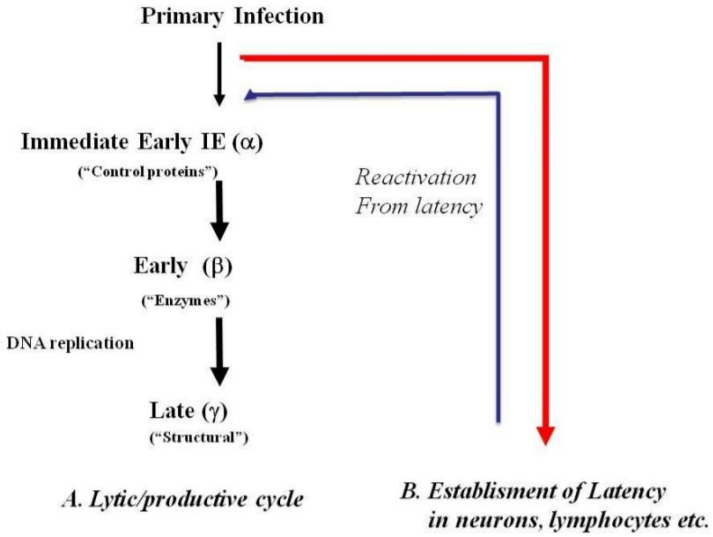

***Figure 5*. Typical herpesvirus life cycle schematic representation.** Adapted from (Riaz, Murtaz-ul-Hasan, e Akhtar 2017).

Balancing between lytic and latent γ-herpesvirus life cycle. Immediate Early (IE), Early (E/) and Late (L/) proteins support herpesviruses lytic/productive cycle (A). A restricted amount of cells allow γ-herpesvirus latency, from which there can be reactivation of the lytic way.

It seems that herpesviruses have evolved and exploiting multiple cell entry pathways based on target cell type receptors (Spear e Longnecker 2003). Two entry pathways have been identify: virus endocytosis, followed by fusion of the viral envelope with the host cell endosome membrane (Shukla e Spear 2001), and viral glycoproteins binding to host cell surface receptors, inducing a precise events sequence of attachment, activation and fusion between viral envelope and cell membrane (Sathiyamoorthy et al. 2017). To date, it is believed that herpesviruses host cell entry mainly depends upon viral envelope glycoproteins interaction with cellular surface receptors. Differently from the other enveloped viruses, that usually exploit a single glycoprotein to entry the host cell, herpesviruses seem to involve a multiglycoprotein entry mechanism. Each herpesviral glycoprotein could potentially interact with several cell receptors or glycoproteins, undergoing and inducing conformational changes to favor membranes fusion, thus explaining the complexity of herpesvirus entry processes (Connolly et al. 2011). Cellular receptors can be distinguished into "binding" and "entry" receptors, responsible for viral particles accumulation on cell surface and envelope-cell membrane fusion, respectively. In particular, BoHV-4 gB envelope glycoprotein interacts with the heparan sulfate binding receptor to attach cell surface, bringing cell

membrane and viral envelope into close apposition, while the gB-gH-gL complex mediate their fusion and subsequent virus entry (Spear e Longnecker 2003).

BoHV-4 gB-gH-gL complex represents the so called "core fusion machinery" and is highly structurally conserved among herpesvirus subfamilies. However, it has been observed that, in some cases, different herpesviruses, seem to interact with several non conserved cell accessory proteins in order to trigger membranes fusion (Heldwein e Krummenacher 2008; Stampfer e Heldwein 2013). Epstein Barr Virus (EBV), for example, exploit gp42 as additional protein.

Specifically, the gH-gL heterodimer, by interacting to host cell receptors, both directly then indirectly thanks to additional viral proteins and accessory cell receptors, provides the virus-specific tropism (Spear 2004). It has been observed that BoHV-4 gp8 fortifies viral interaction to cellular heparan sulfates (Vanderplasschen et al. 1993), but wheather other specific cellular accessory receptors are involved into virus attachment is not clear yet. During fusion, gH-gL heterodimer is responsible for gB conformational changes and its cell plasma membrane penetration, thus drawing viral and host cell together and inducing pores formation into plasma membrane, through which viral contents and host cytoplasm get mixed. During this step, viral nucleocapsid and tegument proteins are released into host cell cytoplasm, across which they reach the Nuclear Pore Complexes (NPCs) by migrating on microtubules skeleton (Riaz, Murtaz-ul-Hasan, e Akhtar 2017). During migration, tegument proteins participate in capsids transportation to the host nucleus (Ibáñez et al. 2018): some outer tegument proteins dissociate from viral nucleocapsid and induce cell metabolism modifications, while inner tegument proteins remain virus associated until it reaches NPCs (Granzow, Klupp, e Mettenleiter 2005). At this point, the viral genome is released within the host nucleus, together with the α-TIF (α-gene Trans Inducing Factor) tegument proteins, where it circularizes before latency or lytic proteins synthesis (Weller e Coen 2012; Riaz, Murtaz-ul-Hasan, e Akhtar 2017).

Based on several factors, such as cells susceptibility and permissiveness, BoHV-4 is able to establish different kind of infections. Presence and type of cell surface receptors determine cell susceptibility and virus attachment and entry, while on cell permessiveness depends if the virus will begin a lytic or latent infection.

Lytic cycle

Although herpesviruses differ in many several aspects, their DNA replication mechanism during lytic infection is highly conserved (Weller e Coen 2012). As well as encapsidation, DNA replication, takes place into specific globular compartments that are generated by host cell nucleus re-organization after virus infection (Weller 2010). These compartments are responsible for the herpesvirus infection pathognomonic sign of basophilic nuclear inclusion bodies appearence. Moreover, chromatin margination and cell surface rounding are as well characteristic signs of herpesvirus infection (*Encyclopedia of Microbiology* 2009).

2-4 hours post-infection, viral tegument proteins induce IE genes transcription by interacting with the host transcriptional machine and inducing host RNA Polymerase II activity. BoHV-4 IE1 and IE2 genes products, once synthetised into cytoplasm, migrate back to the nucleus to block IE genes transcription and favor E and subsequently L genes expression. Several E genes encode for enzymes, such as DNA polymerase, SS-DNA binding proteins, ribonucleotide reductase and TK, that are involved into viral genome replication, while other E genes encode for proteins that clusterize at the *oriLyt* position of viral genome. It has been proposed that herpesviruses DNA synthesis is started as a theta mechanism and, at some point during replication, it is switched to a rolling cycle mechanism, which represents the herpesvirus predominant replication mechanism and leads to long viral genomic DNA concatamers formation. Once viral DNAs are newly synthetized, they serve as template for L genes transcription by E enzymes. L mRNAs, are translated on cellular endoplasmic reticulum (ER) into structural proteins, necessary for virions assembly. Subsequent viral DNAs cleavage and packaging represent one tightly coupled process that take simultaneously place during capsids assembly (Riaz, Murtaz-ul-Hasan, e Akhtar 2017). The exact mechanism through which viral assembly and maturation stages proceed from the host nucleus to the host cytoplasm, where they are completed (Thellman e Triezenberg 2017), has been studied for a long time (Mettenleiter 2004). To date, the most accepted theory is called the "envelopment, de-envelopment, re-envelopment pathway", proposed by Stackpole (Stackpole 1969; Campadelli-Fiume 2007; Bigalke e Heldwein 2016). Stackpole model mechanism supposes that, the first "envelopment" step, starts with capsids budding at the inner nuclear membrane, while the subsequent "de-envelopment" step takes place in the perinuclear space, where viral particles fuse their membrane with the outer nuclear membrane, reaching the host cytoplasm. Once in the cytoplasm, naked capsids move on microtuboles tracks toward trans Golgi network (TGN) and early endosomes (EE) vescicles (David C. Johnson e Baines 2011), where the final "re-envelopment" step takes place, during

which capsids surface is structurally completed with tegument layer and lipid envelope (Bigalke e Heldwein 2016). Finally, TGN and EE vescicles containing the mature enveloped virions reach the cellular plasma membrane where the viral progeny is released by the cell by exocytosis (*Fig.6*) (Riaz, Murtaz-ul-Hasan, e Akhtar 2017).

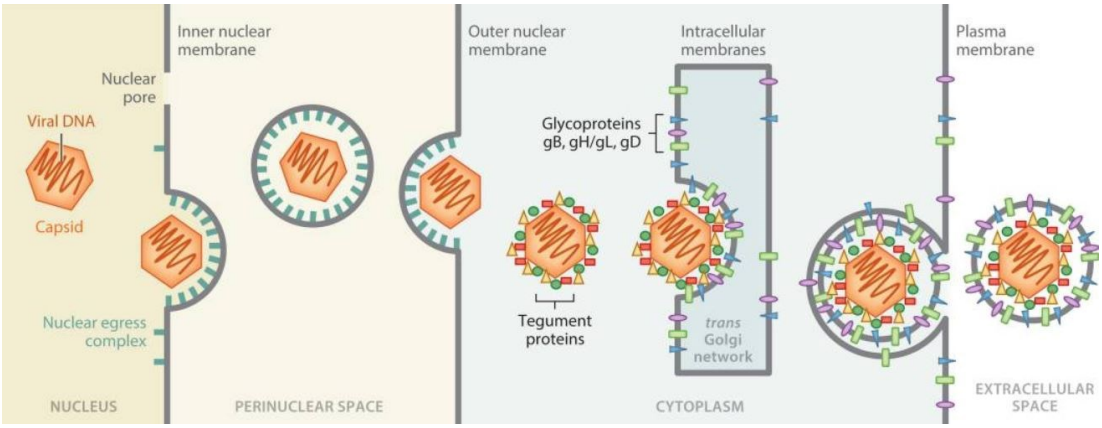

***Figure 6*. Schematic representation of the "envelopment, de-envelopment, re-envelopment pathway" proposed by Stackpole.**

Adapted from (Bigalke e Heldwein 2016)

Starting from the “envelopment”, where viral capsids budd at the inner nuclear membrane, proceeding with the "de-envelopment" into the perinuclear space, where viral particles fuse their membrane with the outer nuclear membrane, reaching the host cytoplasm for the "re-envelopment", where capsid are enriched with tegument layer and lipid envelope and ending with viral progeny release by exocytosis.

As virions production and release are generally followed by host cell irreversible damages, viral lytic cycle is indirectly responsible for herpesviruses pathogenesis (Guo et al. 2010).

As well as for all the other herpesviruses, BoHV-4 productive cycle is associated to viral progeny release and cell destruction. BoHV-4 exibits a slow replication cycle, characterized by the induction of Cythopatic Effect (CPE) 48-72 hours post-infection, defined by the presence of rounded cells spreading near plaques with irregular contours on *in vitro* cells monolayer (*Fig.7*) (Bartha, Juhász, e Liebermann 1966).

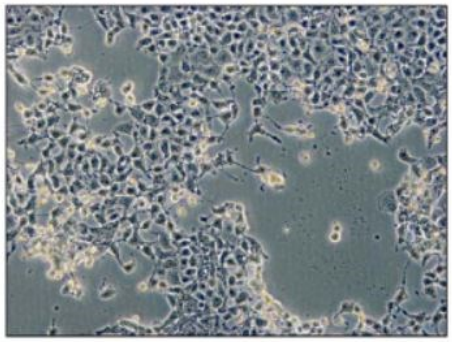

Figure 7. **Typical BoHV-4 Cythopatic Effect (CPE).**

Adapted from (Gaetano Donofrio, Franceschi, Capocefalo, Cavirani, et al. 2009)

Phase contrast microscopic image (10X) of typical BoHV-4 Cythopatic Effect (CPE) plaque formation on *in vitro* Bovine Embryo Kidney (BEK) cells monolayer, 48 hours post-infection.

48-72 hours post-infection, it is possible to observe BoHV-4 typical cytoplasmic inclusion bodies budding from the Golgi network, in which structured viral particles at different maturity stages are accumulated. Based on the fact that inclusion bodies formation is also typical of cytomegaloviruses lytic cycle, in the eighties, BoHV-4 has been erroneously classified as a β-herpesvirus (Storz et al. 1984). Depending on cell type and viral Multiplicity Of Infection (M.O.I.), BoHV-4 *in vitro* infection usually determines cell culture total lysis after about five days of incubation (Markine-Goriaynoff et al. 2003).

γ-herpesviruses relatively little pathogenicity is relegated to their lytic cycle, which is started only on rare occasions, usually due to cellular stress events. BoHV-4 lytic cycle resume is controlled by three IE trans-activating proteins: the ORF50-encoded IE2 and ORF57-encoded IE1 proteins, both well conserved among γ-herpesviruses, and the BoHV-4 specific Bo4 and spliced Bo5-encoded protein. These IE proteins induce strong viral genomic DNA synthesis activation and massive viral progeny generation and release from infected cells (Ackermann 2006).

Latency

During this life cycle stage, herpesviral genome is maintained as a circularized unintegrated episome within the host cell nucleus while cellular nucleosomes appear more dense and relatively more stable. Latency is characterized by no replication process and, considerably varying among herpesviruses, by limited or no genes expression (Riaz, Murtaz-ul-Hasan, e

Akhtar 2017). It has been observed that, despite viral DNA is closely associated to particular histones and strictly controlled by the cellular epigenetic mechanism, it expresses a small set of viral proteins and non coding RNAs, known as "Latency Associated Transcripts" (LATs) (Thellman e Triezenberg 2017). LATs are belived to be involved into the establishment of the delicatly -balanced equilibrium through which herpesviruses limit the host immune system responses, mantaining a long term persistance. Contrarily to what happens during the lytic state, where herpesviruses exploit their own viral DNA polymerase, during latency, LATs are transcribed by the cellular DNA polymerase (Grinde 2013). Interestingly, Ahmed et al. (Ahmed et al. 2002) reported that HSV-1 LATs are the only transcripts that can be frequently detected during viral latency. HSV-1 LAT gene doensn't encode any protein, but it contains a 2Kbp region which is involved into apoptosis ihibition, mantaining latency through IE transcripts down-regulation and promoting infected neurons survival. The lower lytic genes expression of latently infected cells, seems to be directly correlated to high miRNAs presence, which over 50% are encoded by HSV-1 LAT region.

Herpesvirus subfamilies common feature consists of being able of entering into a latency state when conditions are not adequate to support their lytic cycle. Herpesvirus thus can establish lifelong persistent infections into host organisms even after a secondary infection (Etienne Thiry, Dubuisson, e Pastoret 1986). Differently from α- and β-herpesviruses, γ-herpesviruses seem to predilige latency over lytical replication (Ackermann 2006). However, herpesviruses differ for the mechanisms through which they establish latency and the stimuli by which they are *in vitro* re-activated. Latency entry and maintenance are supported and regulated by different gene patterns expression among herpesviruses; some of them exploit a specific protein pattern all through latency duration, while others involve multiple proteins patterns or do not even express any proteins. In EBV latently infected B cells, for example, based on cell differentiation stage, three distinct LATs patterns are produced (Babcock, Hochberg, e Thorley-Lawson 2000). Based on which viral transcripts are present into the infected cell, EBV latency can be classified into three different latency types, I, II or III. Type I latency is tipically present in Burkitt's lymphoma, type II in Hodgkin's lymphoma, T cells lymphomas and nasopharyngeal carcinoma, while type III is associated with B cells lymphomas and EBV infected B cells. However, it is not yet understood what stimuli trigger *in vivo* viral lytic cycle reactivation (Riaz, Murtaz-ul-Hasan, e Akhtar 2017).

Regardless the gene pattern type exploited, LATs expression is assured by an *ORI* present on the viral episome, different from the *ORIlyt* employed during the lytic cycle (G. Donofrio, Cavirani, e van Santen 2000). Moreover, it has been observed that the same herpesvirus can assume different latency behaviours depending on animal species and infected cell type (Etienne Thiry, Dubuisson, e Pastoret 1986). γ-herpesviruses have been shown to express several latency-associated proteins on latently infected cells plasma membrane, playing a crucial role on both viral and cellular gene expression modulation, favoring cells growth but without interfering with the latency state of the resident virus (Ackermann 2006). The γ-herpesviruses Latency-Associated Nuclear Antigen (LANA) protein is encoded by ORF73 and seems to play a key role into latency manteinance (Riaz, Murtaz-ul-Hasan, e Akhtar 2017). γ-herpesviral latent genome persists with an episomal form inside the host nucleus of infected cells, where LANA protein uses its N-terminal to bind the host cellular chromatin while its C terminal interacts with different prDNA sequences, starting viral replication and contemporarly allowing viral episome anchorage to host chromosomes. This mechanism guarantees viral episomes distribution among the newly formed cells during cellular division in rhadinoviruses. ORF73 orthologues genes are present among all rhadinoviruses and BoHV-4 present the shorter one. Thirion et al. (Thirion et al. 2010) showed that ORF73-deletetion doesn't affect BoHV-4 *in vitro* replication but compromises its *in vitro* persistence into latently infected cells cultures and its *in vivo* capability to latently infect a rabbit model. Therefore, BoHV-4 ORF73 is indispensable for *in vivo* virus persistence. Thus, viruses replication success is achieved through a delicate evolutionary interactions equilibrium between the pathogens and the host organisms. The ability to infect a broad range of different host cells types and species plays a prompting role in viral replication success. Viral host range depends upon susceptibility and permissiveness of targeted cells. The fact that γ-herpesviruses predilige latency over the lytic cycle does not mean that they are not capable of infecting a host cells broad spectrum (Ackermann 2006).

On the contrary, BoHV-4, in addition to its natural host, is able to infect and replicate, both *in vitro* and *in vivo*, into a various host species range (L. Gillet et al. 2004).

In vitro Host range

BoHV-4 replication and CPE have been in vitro detected in several bovine and other animal species cell lines (Gaetano Donofrio e van Santen 2001). Its broad host cells spectrum might be due to its interaction with the cellular heparan sulfate receptor, located on almost all

vertebrate cells surfaces (Vanderplasschen et al. 1993). Since its discovery, BoHV-4 infectivity and tropism have been *in vitro* studied on several cell types and different specific primary and continuous cell lines susceptible to BoHV-4 infection have been identified. It has been seen that BoHV-4 can replicate in both bovine primary cell lines (such as kidney, lungs, spleen, testes, skin and thyroid bovine primary cell lines, Embryo Bovine Tracheal cells (EBTr), lymphosarcoma calf thymus and fetal bovine bone marrow) and immortalized ones (among which: Madin Darby Bovine Kidney (MDBK) cells, Georgia Bovine Kidney (GBK) cells, Bovine Embryonic Kidney (BEK) cells and Embryonic Bovine Lung (EBL) cells) (Markine-Goriaynoff et al. 2003). Several experiments demonstrated that BoHV-4 is also able to infect bovine immune system cells, such as histiocytes, thymocytes, T- and B-lymphocytes and macrophage derived cells as proved by Donofrio et al. (Gaetano Donofrio e van Santen 2001), as well as bovine mammary gland, endothelial, tracheal and nasal turbinate cells. Donofrio et al. proved that both epithelial and stromal cells of bovine endometrium are susceptible and permessive to *in vitro* BoHV-4 replication, with the presence of non apoptotic CPEs (Gaetano Donofrio, Herath, et al. 2007). Lin et al. *in vitro* observed that Bovine Arterial Endothelial (BAE) cell line, derived from bovine carotid arteries, is 100-1000 times more permessive to BoHV-4 replication respect MDBK cells, commonly employed for BoHV-4 propagation (Lin et al. 1997). Egyed et al. have isolated a carotid arterial endothelial primary cell line from a BoHV-4 infected rabbit with vascular lesions and demonstrated its subsequent susceptibility to BoHV-4 infection (László Egyed e Baska 2003).

Cells from many animal species (like sheep, goat, buffalo, mink, guinea pig, rabbit, swine, cat, dog, lion, chicken, mouse, ferret, hamster and monkey) have been experimentally infected and showed different level of susceptibility to BoHV-4 infection (László Egyed 1998; L. Gillet et al. 2004). Although BoHV-4 is not considered a neurotropic virus, it has been isolated also from bovine nervous tissues (Yamamoto et al. 2000; Asano et al. 2003). Donofrio et al. investigated the *in vitro* interaction between two reporter genes recombinant BoHV-4-based vectors (BoHV-4EGFPΔTK and BoHV-4/26A3neo) with the Neuroblastoma (N2a) cell line. Their data demonstrated that BoHV-4 is able to persistently infect N2a cell line, producing viable viral particles, but without interfering with cellular differentiation into specific neuron-like cells (Gaetano Donofrio, Grandi, et al. 2004).

Moreover, *in vitro* BoHV-4 infection has also been tested on human cell cultures. 21 human cell lines have been *in vitro* tested for their sensitivity and permissiveness to BoHV-4 infection, revealing that human cell lines from myeloid and lymphoid origins were resistant

to infection, while epithelial, carcinoma or adenocarcinoma cells from various organs were susceptible but poorly permissive to BoHV-4 replication (L. Gillet et al. 2004). Donofrio et al. were able to *in vitro* establish a BoHV-4 persistently infected Human Rhabdomyosarcoma (RD-4) cell line, through the use of a recombinant BoHV-4 carring a neomycin resistance selective gene. RD-4 persistently infected cells produced viable viral particles at all passages tested, but grew more slowly compared to uninfected cells, thus not showing growth-transformation capacity (G. Donofrio, Cavirani, e van Santen 2000).
Despite its association to cancer remain unclear, there are data showing BoHV-4 viro-oncoapoptosis ability on some human carcinomas cell lines, both *in vitro* and *in vivo* (Laurent Gillet et al. 2005). Based on these experiments, a recombinant BoHV-4-based vector has been generated for a viro-oncoapoptosis trial on animal models against human glioma (Redaelli et al. 2010). These data might suggest a BoHV-4 potential ability to establish infections in humans, and since BoHV-4 is massively prevalent in cattle populations, there could be many potential transmission route to humans. However, BoHV-4 cross-species transmission to humans is not possible, because of the presence of natural antibodies-complement dependent in human sera, able to efficiently neutralize BoHV-4 (Machiels et al. 2007).

In vivo Host range

Considering its extended worldwide distribution, BoHV-4 isolates have been identified from many different animal species sera and tissues, as well as from its natural host.
Many isolates have been recovered from non-bovine ruminants, like sheep (*Ovis aries*), goat (*Capra hircus*), American bison (*Bison bison*), zebu (*Bos indicus*), and African buffalo (*Syncerus caffer*) (Todd e Storz 1983; Moreno-Lopez et al. 1989; Rossiter et al. 1989; L. Gillet et al. 2004). Interestingly, it has been observed that BoHV-4 infection can protect African buffalo from malignant catarrhal fever (Rossiter, Gumm, e Mirangi 1988).
400 sera collected from wild African Buffoaloes, from different geographical areas, were tested positive for BoHV-4 presence with 70% of serum prevalence, much higher if compared to cattle. These data suggested that the African Buffalo might represent BoHV-4 original host specie (Benjamin Dewals et al. 2005; B. Dewals 2006). Sporadic isolations from non-ruminant species, like lion, cat and owl monkey (*Aotus trivirgatus*), were also reported.

Moreover, even if it was not isolated on field natural conditions, other non-ruminant species, like guinea pigs and rabbit, also showed to be susceptible to BoHV-4 infection. These two species, together with the goat, are experimentally employed as proper animal models for BoHV-4 biology studies (Laurent Gillet et al. 2005; L. Gillet et al. 2005). Among these models, because it succesfully supports BoHV-4 infection, rabbit is considered a good experimental model also for BoHV-4 pathogenesis studies (L. Egyed, Kluge, e Bartha 1997; László Egyed e Baska 2003). Interestingly, since anti-BoHV-4 antibodies have been found in Asian elephants (*Elephas maximus*) and a DNA polymerase strictly related to the one of BoHV-4 has been identified in black rhinoceros (*Diceros bicornis*), a BoHV-4 strain circulating between exotic animals might exist (Ae et al. 1990).
All these evidences, higlight the fact that BoHV-4 represents an exception to othe gamma herpesviruses, whose life cycle is restricted to their natural host species (Laurent Gillet et al. 2005; L. Gillet et al. 2005).

BoHV-4-based vector

Its complete genome sequence and its genome manipulation as a Bacterial Artificial Chromosome (BAC), opened the way to the BoHV-4 potential use as a therapeutic gene delivery viral vector. BAC cloning and prokaryotic recombination technology are easier and faster approaches compared to classical homologous recombination in eukaryotic cells, that allow large herpesvirus genomes manipulation and recombinant herpesviral vectors generation (L. Gillet et al. 2005). Particularly, several recombinant BoHV-4s have been generated and exploited as γ-herpesviruses biology models for applied research and as antigens expressing vectors for vaccination or oncolitical purposes. BoHV-4, indeed, possesses several characteristics that value its great potential as viral vector:

- Its genome organisation is less complex if compared to other herpesviruses, thus making it easier to be manipulated for either transgenes insertion or viral ORFs deletion. Moreover, additional foreign genetic sequences of at least 50 Kbp can be stably inserted into its genome (L. Gillet et al. 2005).
- Differently from most γ-herpesviruses, it can both *in vitro* and *in vivo* replicate into a wide range of host species as it is able of targeting different cell types, and it easily *in vitro* propagate in cell cultures, thus being easily amplified, without interfering with cell differentiation. Moreover, it has been seen that transgene expression is mantained in both

undifferentiated and differentiated infected cells (E. Thiry et al. 1992; Gaetano Donofrio et al. 2002).

- Even if it is recognized as a potential secondary pathogen in cattle affected by uterine diseases (Gaetano Donofrio et al. 2005), there are no evidences of BoHV-4 pathogenicity neither in experimental or natural hosts (E. Thiry et al. 1992). Moreover, despite some cellular genes homologs, capable of influencing cell growth properties, are present in its genome, BoHV-4 doesn't show any growth transformation activity (Zimmermann et al. 2001; Gaetano Donofrio, Cavirani, et al. 2006). These features represent indispensable characteristics for viral vectors safety.
- Although in some cellular lines BoHV-4 infection has been shown to inhibit apoptosis, on the contrary, in some other cell lines, expecially in cancer cells, it seems to favor the process (Gaetano Donofrio, Cavirani, et al. 2006). This feature could be exploited for cancer treatment in order to selectively induce apoptosis of tumoral cells. For example, Redaelli et al. *in vitro* tested the striking tropism and permissive replication on mouse, rat and human glioma cell lines of a recombinant BoHV-4 armed with the herpes simplex virus type 1 thymidine kinase suicide gene in combination with the pro-drug Ganciclovir (GCV), proving its efficacious cells killing by inducing apoptosis. This combined armed BoHV-4/GCV therapy also demonstrated a significant *in vivo* survival increasing in immunocompetent orthotopic syngenic mouse and rat glioma models. Moreover, they also found out BoHV-4 capability of *in vivo* selectively infect glioma cells only, without causing pathogenicity in the rat brain. Despite it is not known as a naurotropic virus, BoHV-4 has been frequently found in both peripheral and central nervous system during persistent infections.Thus, all these data, suggest the idea of exploiting BoHV-4 as a gene delivery vector for the nervous system and an alternative potential oncolytic virus for glioma treatments (Gaetano Donofrio et al. 2002; Redaelli et al. 2008; 2012).
- Since it can establish persistent infections both in experimental and natural hosts, recombinant BoHV-4s can be exploited for long enough time transgenes expression to trigger an efficient host immune response. This feature represents an important requisite for viral vectored-based vaccines effectiveness (Lomonte et al. 1996). Particulalrly, since macrophages and monocyte cell lines represent BoHV-4 persistent infection site, the anti-viral immune response is amplyfied by antigens processing and presentation (Gaetano Donofrio et al. 2005; Gaetano Donofrio, Cavirani, et al. 2006).

- In some cases, it naturally establish non-replicative persistent infections, with three weeks transgene expression and no viability compromission of the host cells, thus recombinant BoHV-4s can be exploited as immunogenic and replication incompetent viral vectors (L. Gillet et al. 2005).
- Although it has not shown neither pathogenity or transforming activity, BoHV-4 could also be further attenuated, in term of replication, by deleting the L1.7 gene, coding for the 1.7Kbp poly-adenylated RNA, from its genome (Antonio Capocefalo et al. 2013).
- Importantly, since it *in vivo* elicits low or no serum neutralizing antibodies levels in hosts, BoHV-4 can play a dual role of both acting as an effective viral vector and as an adjuvant (Gaetano Donofrio, Cavirani, et al. 2006).

From its sequencing, BoHV-4 genome and biology knowledge have improved improved a lot. All its mentioned features suggest it as a safe and effective viral vector, making real the possibility to exploit it in vaccine and gene therapy fields. BAC homologous recombineering technology has been widely exploited from Donofrio et al., successfully producing different BAC-cloned BoHV-4-based vectors, employied in experimental vaccination and oncolysis researches.

Recently, two recombinant BoHV-4-based vectors delivering Nipah Virus (NiV) attachment (G) and fusion (F) surface glycoproteins, BoHV-4-A-CMV-NiV-G-ΔTK and BoHV-4-A-CMV-NiV-F-ΔTK respectively, have been generated as a potential vaccination platform to protect animals and humans against NiV-caused respiratory disorders and encephalitis. The effectiveness of the induced immunities has been experimentally studied in pigs and compared to the control Niv G-recombinant canarypox (ALVAC) vector, that has previously demonstrated to be protective in pigs. Both recombinants were able to induce specific antibody responses, with Niv G-recombinant induced neutralizing antibody titers comparable to ALVAC but greater compared to Niv F recombinant induced ones, and specific CD4+ and CD8+ T cell mediated immunities, particularly effective in BoHV-4-A-CMV-NiV-G-ΔTK immunized pigs (Pedrera et al. 2020). Also the recombinant BoHV-4-based vector delivering the Peste des Petits Ruminants Virus (PPRV) hemagglutinin (H) surface glycoprotein, BoHV-4-A-PPRV-H-ΔTK, generated in this PhD thesis project, showed an enhanced capability to induce both strong humoral and cellular immune responses in immunocompetent inoculated mice (Macchi et al. 2018).

Two different recombinant BoHV-4s have been generated and successfully experimented in the mammary cancer field. The recombinant BoHV-4-based vector, delivering an epidermal growth factor receptor 2 (HER-2) isoform, has experimentally shown to break tolerance and effectivily protect HER-2 transgenic BALB-neu T mice by eliciting a highly protective anti-mammary cancer cell antibody response (Jacca et al. 2016). Another recombinant BoHV-4-based vector, delivering a mouse isoform of the cysteine-glutamate antiporter (xCT/SLC7A11) protein, over-expressed in mammary Cancer Stem Cells (CSCs), demonstrated to be able to induce both an efficient humoral response and the effector T lymphocytes clonal expansion, in BALB/c mice. The antibodies were not only specific against the mouse full lenght isoform but also detected and neutralized the protein native conformation. Moreover, in addition to mammary cancer growth decrease, vaccinated mice showed lung metastases prevention and reduction of metastases from pre-existing tumor masses (Gaetano Donofrio et al. 2018).

Moreover, recombinant BoHV-4s, expressing antigens of A category agents, such as Monkeypox (MPX) and Ebola (EBO) viruses, have also been exploited, showing the possibility of a therapeutic intervention in human zoonosis, avoiding pathogens direct manipulation. A small challenge study with a recombinant BoHV-4, BoHV-4-A-EF1α-M1R-gD106-ΔTK, expressing the MPXV M1R glycoprotein, was performed on STAT1 knockout mice. Intraperitoneally inoculated STAT1(-/-) mice showed no weight loss or adverse events, and a 100% protection against MPXV mortality and morbidity (Franceschi et al. 2015).Goats immunized with a recombinant BoHV-4, BoHV-4-syEBOV-gD106-ΔTK, expressing EBOV surface immuno-dominant glycoprotein (GP), showed no viremia or secondary virus localization, and produced an elevated, 6 months-lasting, specific antibody immune response (Rosamilia et al. 2016).
All these experimental works, based on recombinant BoHV-4s, corroborate this γ-herpesvirus as a potential, validate vaccine platform.

BAC-BoHV-4

Bacterial artificial chromosomes are fertility - (F-) factor-based plasmids, able to stably replicate in low copy numbers. Contrary to multi-copy plasmids, that allow relatively small insert sizes up to 50 Kbp, BACs represent the vectors of choice for large genomic fragments, up to 300 Kbp, cloning and manipulation. Since genomic insertions may contain several

regulator elements, such as gene specific extragenic cis-regulatory elements (promoter, terminator and enhancers), BACs can leave the cloned gene expression under its own regulatory elements control, thus mimicking its endogenous expression pattern. This feature lend BACs as the ideal choice for transgenic mice generation. The ease in obtaining BAC clones and the possibility of stably clone methylated eukaryotic DNAs, coupled with high transformation efficiency and simple high-quality DNA purification protocols, make BAC plasmids an aternative and valid resource to conventional vector systems for genome functional studies and transgenes expression under heterologous promoters control (Sharan et al. 2009). In the late 1990s the introduction of the recombination-mediated genetic engineering (recombineering) technique, allowed the overcoming of BAC large size limitation, and its much more flexible manipulation, comparing to conventional cloning and mutagenesis, in order to precisely intruduce or delete one or more nucleotides or DNA sequences (Narayanan e Chen 2011). Because of its elevated efficiency and small homology requirements, recombineering can be widely exploited for a wide range of applications and it nowadays represents the most routinely exploited subcloning technique (Sharan et al. 2009). Recombineering is an *in vivo* genetic engineering technique, adapted from bacteriophages, where genes responsable for homologous recombination have been carefully moved on mobile plasmids, such as BACs, and subsequently transfered to *Escherichia coli* host strains containing the coliphage λ Red system or the RecET system from the Rac prophage. Recombineering strategies key feature is that DNA manipulation is indipendent from *E coli* endogenous homologous recombination functions, thus this method can be used to transiently introduce recombination proficiency into recombination deficient hosts. Transient recombineering systems expose target DNAs for just a limited short time to the recombination enzymes, thus facilitating DNA substrates stable modifications with no or low risks of rearrangements (Narayanan e Chen 2011). Target DNAs, providing the homologous substrates, designated as "targeting constructs", are introduced through electroporation into recombinant *E coli* hosts, in order to induce the desired genetic chances. Differently from conventional homologous recombination methods, recombineering, allows the targeted genetic change at any position on the BAC plasmid, just needing short 50 bp homology arms (Sharan et al. 2009). The BAC-BoHV-4 recombineering achieved in this PhD thesis project has been obtained through the DH10B-derivative engineered SW102 *E coli* strain, carring the λ Red bacteriophage recombination system and the galactokinase (GalK) gene deletion (Warming et al. 2005). A specific "A-pathogenic" BoHV-4 strain, named BoHV-4-A, isolated from the milk cellular fraction of a healthy cow, has been

exploited in order to generate a BAC-BoHV-4 with no pathogenic risks (Antonio Capocefalo et al. 2013). λ Red bacteriophage system includes the phage recombination genes gam, bet and exo, under the *lac* promoter trascription control. Gam gene product, *Gam*, prevents the *RecBCD E. coli* nuclease from linear DNA fragments degradation, guaranteeing the transformed linear DNA *in vivo* preservation. Bet gene encodes for the ssDNA binding *Beta* protein that promotes the annealing between the two complementary DNA molecules, while exo gene product, *Exo*, possesses a 5′ to 3′ dsDNA exonuclease activity. Together, these last two proteins allow the precise, desired linear DNA isertion, inducing the creation of the expected genetic recombinants. *Exo* protein is thought to degradate dsDNAs from both 5' ends, allowing *Beta* binding to the exposed DNA single strands. Several recombineering models suggest that the *Beta*-bounded single strands regions of the incoming linear DNAs get annealed to complementary single stranded gaps locally arising at the replication fork during DNA replication. This recombineering model is consistent with the fact that an oligo annealing to a discontinuosly replicated single strands would lead a higher recombination rate respect its "leading strad"-complementary oligos. *In vivo* DNA engineering needs a short but high-level pulse inducing λ Red proteins temporally limited expression, in order to minimize Gam protein toxic effects and possibly avoid undesired genetic rearrangements. λ prophage system expression is restricted to its uniquely strong endogenous regolatory system, which represents the natural strategy through which it regulates its own recombination functions. The cI857 λ repressor cooperatively binds two sets of operator sites located at both pL and pR promoters. These two repressors-bound operator sets interact to each others, creating a tight proteic handcuff impeding the genes expression. Both operator sets and the repressor gene are present in the SW102 strain. The repressor is temperature sensitive, being active at 30-34°C and inactivated when shifting temperature up to 42°C. By shifting the bacterial culture temperature to 42°C for 15 minutes, the repressor get rapidly inactivated and λpL promoter induces recombination genes high levels expression. The short 15 minutes induction time minimizes undesired cellular stress and accidental recombinations. After that, subsequent temperature lowering induces repressor renaturation and recombination genes repression restoration (Sharan et al. 2009).

In SW102 strain, λ Red recombination genes are flanked by the Biotin and Galactose Operon systems, exploited for the identification and metabolic selection of recombinant positive colonies (Warming et al. 2005). The BAC recombineering system has been exploited in order to facilitate BoHV-4 genome manipulation. The Bo2-Bo3 ORFs intergenic region, located at BoHV-4-A genome left-end, has been used as the target site for the BAC cassette

insertion. BAC cassette contains an Enhanced Green Fluorescent Protein (EGFP) reporter gene under the CMV immediate early gene promoter transcriptional control and the F1 plasmid elements: chloramphenicol resistance gene (Cam), origin of replication (Ori2) and the repE, parA, parB, parC partitioning protein genes. The cassette is LoxP floxed at both ends and flanked by BoHV-4-A Bo2, Bo3 homologous regions. The BAC-BoHV-4-A generation has been achieved through homologous recombination between a linearized pBo2-EGFP-BAC-Bo3 plasmid and the BoHV-4-A purified genome, co-transfected into Bovine Embryo Kidney (BEK) cells. EGFP fluorescence allowed the infectious reconstituited recombinant virus detection and the subsequent electroporation of its circular recombinant intermediates into the DH10B *E. coli* strain, thus generating the stable pBAC-BoHV-4-A (*Fig.8*) (Gaetano Donofrio, Franceschi, Capocefalo, Cavirani, et al. 2009).

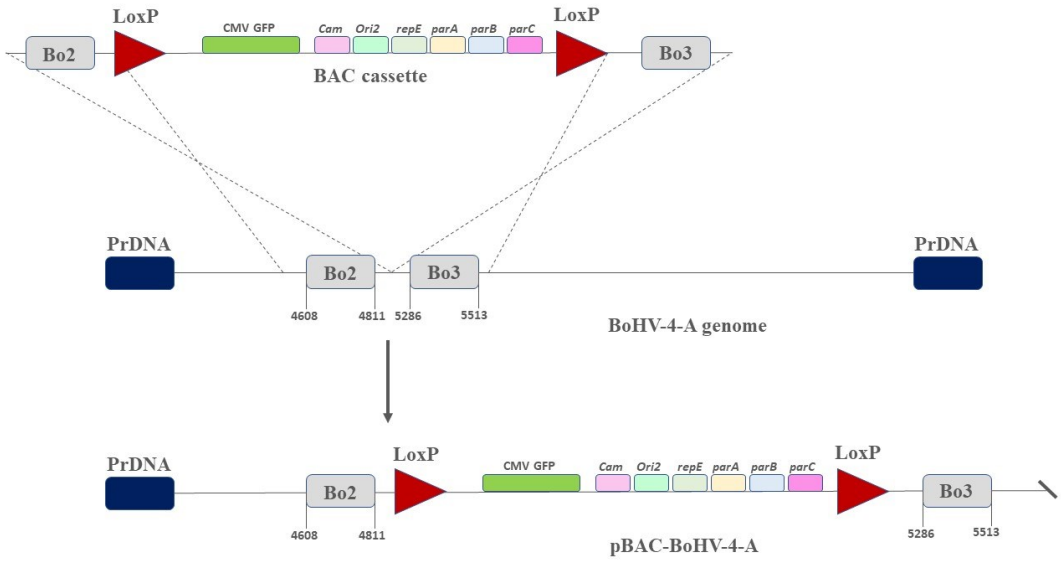

***Figure 8*. Schematic representation of pBAC-BoHV-4-A *in vitro* generation.**

Clonation of BoHV-4-A genome as a Bacterial Artificial Chromosome (BAC): pBAC.BoHV-4-A. Introduction of the LoxP-floxed BAC cassette, containing the CMV-GFP sequence and F1 plasmid elements (Chloramphenicol resistant gene, Cam; F1 origin of replication, Ori2; partitioning protein genes, repE, parA, parB and parC) into the BoHV-4-A genome via classical homologous recombination in eukaryotic cells, exploiting BoHV-4-A Bo2 and Bo3 regions as homology regions.

During a process called "Targeting", the PCR product, Kana/GalK cassette, flanked by two TK homology arms of almost 1Kbp, have been precisely cloned into pBAC-BoHV-4-A exploiting the λ Red-based reecombineering system (*Fig.9*). The TK fragments have served as homologous recombination regions, while the Kana/GalK cassette is essential for the positively recombined clones screening (Gaetano Donofrio, Franceschi, Capocefalo, Cavirani, et al. 2009).

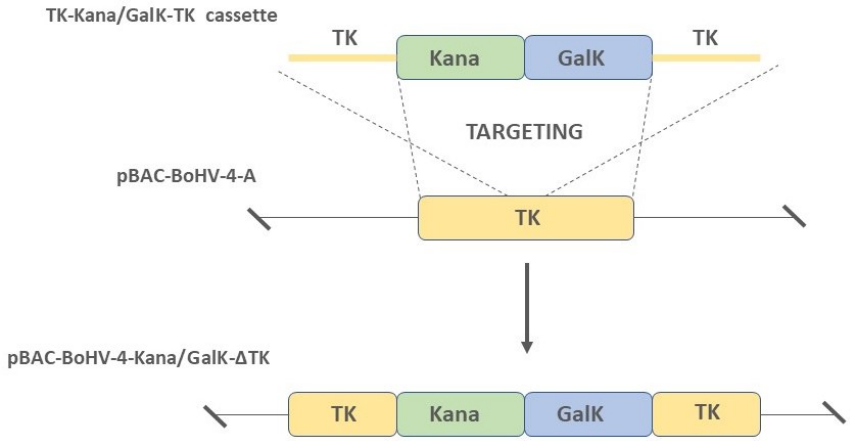

***Figure 9*. Schematic representation of pBAC-BoHV-4-Kana/GalK-ΔTK *in vitro* generation.**
Targeting: introduction of the Kanamycin resistance/Galactokinase (Kana/GalK) cassette into pBAC-BoHV-4-A via heat inducible homologous recombination in SW102 *E coli* strain, using BoHV-4-A Thymidine Kinase (TK) as homology arms.

Positive clones selection is performed on minimal plates, containing galactose as the only carbon source, biotin and leucine to compensate SW102 deficiencies and chloramphenicol for BAC maintenance. SW102 containing the pBAC-BoHV-4-A-Kana/GalK serve as based platform for the generation of recombinant, therapeutic BoHVs. During a second-step process, called "Re-Targeting", the Kana/GalK cassette is replaced with another cassette containing the transgene of interest, flanked by the two same short homology arms. The positively recombined clones are selected through two screening steps: first, a solid phase selection on plates containing the 2-deoxy-galactose (DOG) galactose analogue, that get transformed into the 2-deoxy-galactose-1-phosphate toxic compound when phosphorilated by the GalK enzyme, thus killing non-recombined SW102s, and second, a liquid selective phase in kanamicine (K) and chloramphenicol (Cl) enriched media, discharging all the

K^+/Cl^+ fake positives (Gaetano Donofrio, Franceschi, Capocefalo, Cavirani, et al. 2009). The positively recombined pBAC-BoHV-4-Kana/GalK-ΔTK clones can then be exploited for the recombination of BoHV-4 genome with transgenes of interest. This process is called "Re-Targeting" and it allow the substitution of the Kana/GalK cassette with an expression cassette of interest via the λ Red-based reecombineering system (*Fig.10*).

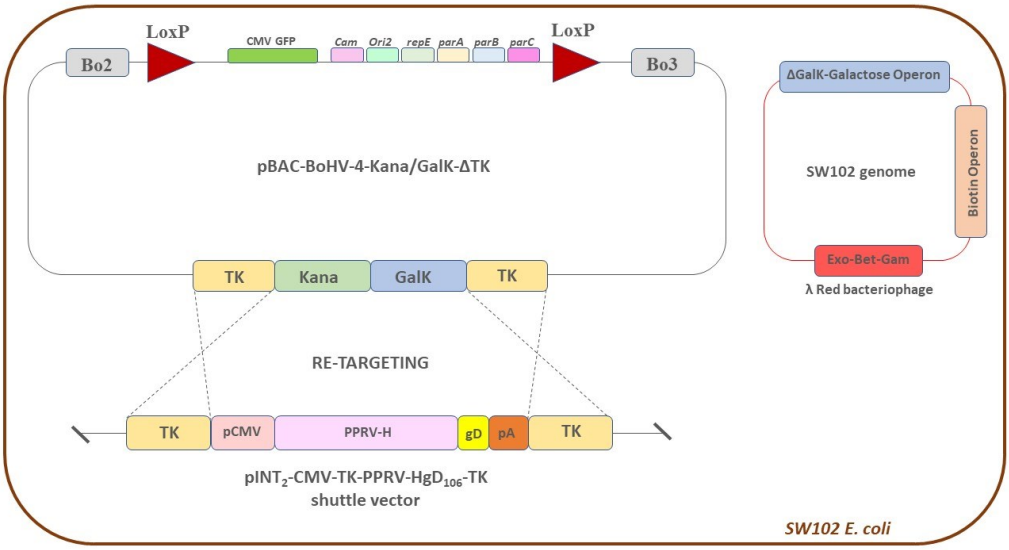

***Figure 10*. Schematic representation of**

Re-targeting: Kana/GalK cassette replacement with the expression cassette containing the transgene of interest, into pBAC-BoHV-4-Kana/GalK-ΔTK via heat inducible homologous recombination in SW102 *E coli* strain, using BoHV-4-A Thymidine Kinase (TK) as homology arms.

This cloning technologies have greatly improved and facilitated recombinant BoHV-4 genome manipulation. The recombinants generation is then accomplished after double checking the transgene cassette insertion through restriction enzyme digestion on agarose gel and southern immunoblotting assay using a specific probe against the insereted sequence. The viable viral particles are obtained by electroporating the purified pBAC-BoHV-4-A-"transgene of interest" into BEK/finCre cells, in which is present the Cre-LoxP recombinase system, capable of exciding the floxed BAC cassette from the viral genome (L. Gillet et al. 2005).

VACCINES

Vaccines are biological preparations aimed to safely stimulate a protective acquired immunity against a specific disease. An ideally effective vaccine should cover important requirements, such as safety, efficacy in terms of high immunogenicity, physical, chemical and genetic stability, ease and low costs of production. Vaccination, since its discovery and first uses in the 10th century, still nowadays represent the most cost effective medical prophylaxis in order to prevent and control infectious diseases (Leitner, Ying, e Restifo 1999) . Nowadays, in human and veterinary medicine, various vaccine types are commercially available and the progressive improvement of molecular biotechnologies allowed the experimentation of new vaccine frontiers in order to overcome conventional vaccination potential problems and optimize their efficiency (Francis 2018).

Conventional vaccines

Conventional vaccines are preparations made of killed or live attenuated pathogenic viruses or bacteria. Their functioning is to mimic as much as possible the structure and behavior of the pathogenic microrganism against which it is desired to induce protection, but without causing disease, in order to induce immunity protection(.Baron, Iqbal, e Nair 2018a; Jorge e Dellagostin 2017; Brun et al. 2008). As the immune system is naturally suitable to optimally recognize and respond against viral and bacterial infections, conventional vaccines aim to mimic the immunity induced by the natural contact with the pathogen. It is indeed not surprising that some of the most effective still nowadays used vaccines are made of live attenuated or killed pathogenic microrganisms. However, there are limitations due to the high costs of vaccine production, increased by the need of multiple administrations to adequately stimulate the immune response. Furthermore, this vaccination is only possible for pathogens that can be *in vitro* cultured (Jain et al. 2015; Jorge e Dellagostin 2017).

Live attenuated vaccines. Live attenuated vaccines have been the first vaccine formulation generated and successfully been used. These preparations are made of live attenuated microorganisms deprived of pathogenic activity but able to stimulate a protective immune response against the virulent ones. Live microorganisms are, indeed, able to infect and replicate into target cells, stimulating strong and long lasting cell-mediated and humoral immune responses, without the need of adjuvants (Brun et al. 2008). However, the need of

adeguate storage, the incomplete immune protection of some bacterial and viral strains and, particularly, safety, due to the high risk of residual pathogenicity, retromutation and virulence reacquisition, or genetic recombination with fully virulent strains («Il Progresso Veterinario - Organo Ufficiale della Federazione Nazionale degli Ordini Veterinari Italiani» s.d.), are concerning problems of these live vaccines (Jorge e Dellagostin 2017; Babiuk et al. 2003; Meeusen et al. 2007).

Inactivated vaccines. Inactivated vaccines preparations are made of killed microorganisms, combined with adjuvants. Inactivated viruses and bacteria usually belong to one or more different strains, and are traditionally inactivated by heat or formaldehyde methods. Compared to live attenuated vaccines, inactivated ones are safer, as there is no risk of reacquiring virulence, and more stable in field natural conditions, as the microorganisms are not alive, and also less expensive to be produced (van Gelder e Makoschey 2012; Jorge e Dellagostin 2017). However, the induced immune response is much less effective than the one induced by live vaccines due to the lack of the microorganism replication and therefore to its inability to stimulate long lasting protection (Cho, Howard, e Lee 2002; Jorge e Dellagostin 2017).
Moreover an additional issue is that, often, viral infections, such as influenza and bluetongue ones, are due to viruses with different serotypes, reducing the vaccine coverage efficacy and requiring a new vaccine formulation to tame new disease outbreacks (Meeusen et al. 2007; Jorge e Dellagostin 2017).

Toxoids vaccines

Toxoids vaccines preparations are made of inactivated bacterial native toxins, named toxoids, combined with adjuvants. Like inactivated vaccines, toxoids are more efficient and safer compared to live ones; however their *in vitro* production impose some limitations, as it is not possible to predict toxoids amount production and as high safety level laboratories are required for some of them to be generated (Arimitsu et al. 2004; Jorge e Dellagostin 2017).

Subunit vaccines

Subunit vaccines are more recent vaccine formulations consisting of specific pathogen proteic particles against which immune protection is desired. Formulations can include more than one protein subunit, in order to allow protection against more than one bacterial or viral strain (Jorge e Dellagostin 2017; Dellagostin et al. 2011). Since these preparations are only made of pathogen particles, no replication occurs and host immune response is specific against those components only, with no risk for safety. Moreover, safety is guaranteed also for the manufacturers as protein subunits are produced in heterologous systems. Another important feature of these vaccines is that protein subunits mantein their native form, thus correctly presenting the antigenic epitopes to the host immune system. However, peptide subunits small size and few specific epitopes, often elicit an incomplete and short-term immune protection compared to live vaccines, where, in addition to antibodies production, there is a strong cell-mediated response (Eshghi et al. 2009; Jorge e Dellagostin 2017; «Il Progresso Veterinario - Organo Ufficiale della Federazione Nazionale degli Ordini Veterinari Italiani» s.d.).

Vector-based vaccines

Vector-based vaccines exploit vectors as antigen/gene delivery systems in order to stimulate the host immune system. The vector itself is usually immunogenic and can be able to deliver multiple antigens. DNA/RNA-based and live vector-based vaccines, all belong to the recombinant vector-based vaccines class (Jorge e Dellagostin 2017).

DNA and RNA-based vaccines. Genetic vaccines formulatons consist of DNA plasmids or RNA messengers engineered with one or multiple genes encoding for antigenic proteins.The interest of using nucleic acids in vaccine formulations began in the early 1990s with the discovery that DNA plasmid inoculated hosts are able to produce an immune response against the plasmid encoded antigens (Wolff et al. 1990; Tang, DeVit, e Johnston 1992; Leitner, Ying, e Restifo 1999). Plasmids use transfected cells transcriptional machinery to express and produce their carried antigenic proteins and stimulate the host immune system through both MHC-I and MHC-II pathways (B. Wang et al. 1998; Leitner, Ying, e Restifo 1999; Jorge e Dellagostin 2017). As well as live vaccines, genetic ones are able to stimulate a strong and long-lasting immunity complete of both humoral and cellular mediated responses, but since only antigenic proteins of the pathogen are expressed, there is no danger of disease onset. However, there are limitations, such as high production costs, mRNAs

instability, and high risk of plasmids integration into the host cell genome (Jorge e Dellagostin 2017).

Live vector-based vaccines. Live vector-based vaccines consist of live attenuated bacteria or viruses, recombined with other pathogens sequences, used as carriers to induce specific immunity against the expressed immunogenic antigens into vaccinated hosts(Jorge e Dellagostin 2017; Brun et al. 2008). Particular attention has been paid on the research and use of recombinant, virulence-free viruses, which have been widely adopted as vaccine platforms much more quickly in veterinary medicine than in humans, where many restrictions still nowadays remain (Draper e Heeney 2010; .Baron, Iqbal, e Nair 2018a; Rollier et al. 2011). The first viral vectored vaccine licensed in human therapy, in 2011, has been a recombinant yellow fever virus expressing two structural antigens of the Japanese Encephalitis virus (Rollier et al. 2011).

VIRAL VECTORED VACCINES

Viral vectored vaccines are chimeric engineered viruses *in vitro* generated (Condit et al. 2016), expressing heterologous antigens from other pathogens, that exploit viruses evolutionary capabilities of entering cells and direct cellular transcriptional machinery in order to express its recombinant genome (M. A. Liu 2010b; Rollier et al. 2011). These vaccines challenge is to minimize the innate and pre-existing adaptive immunity against the immunogenic viral vector itself, in order to maximize the immune response strenght and specificity against the carried antigen. The host immune system has infact evolved adaptive defenses against viral infection, possibly causing a lower expression efficacy of the vaccine antigen and a reduced specific immune response against it. However, at the same time, viral vectors intrinsic immunogenity is an advantage because there is no need of adjuvants addition as they are able to actively stimulate the innate immunity against themself (Ewer et al. 2016).

Infected cells can be eliminated either by $CD8^+$ T lymphocytes and Cytotoxic T cells (CTL) recognizing pathogen-derived epitopes exposed on the infected cell surface via the Major Histocompatibility Complex (MHC) class I or by antibody-dependent lysis or opsonization. Intracellular pathogens, such as viruses, stimulate the induction of the cell-mediated immunity, while extracellular pathogens induce a humoral immunity, but when both pathogen types are on their way to infect other cells, they both can be either lysed or agglutinated by the attack of specific circulating antibodies, or phagocytosed by macrophages or neutrophils. $CD4^+$ T helper lymphocytes activated by the interaction with pathogen-derives epitopes exposed via MHC class II on antigen presenting cells (APCs) surface, can help both antibody and CTL induction. Infected APCs can be directly eliminated by $CD4^+$ as well as $CD8^+$ CTL cells via the induction of different mediators (*Fig. 11*) (Meeusen et al. 2007).

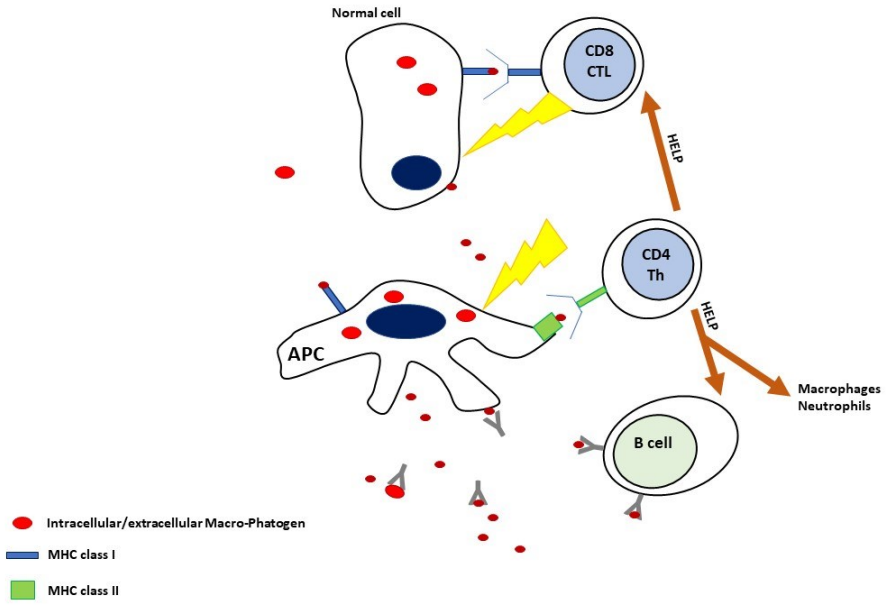

***Figure 11*. Simplified schematic representation of mammals adaptive immune mechanisms against viral pathogens.**

The best example of a recombinant virus based vaccine in veterinary medicine has been the anti-rabies recombinant vaccinia virus-based vaccine. This recombinant vaccine was generated by introducing the rabies virus glycoprotein encoding gene into vaccinia virus genome, obtaining a not pathogenic recombinant vaccinia virus able to elicit a strong anti-rabies neutralizing antibody response in vaccinated foxes, guaranteeing 12 to 18 months immunity(P.-P. Pastoret et al. 1988). This vaccine type has been used for wild animals rabies vaccine prophylaxis in Belgium, France and Luxembourg (P. P. Pastoret e Brochier 1999). Viral based vaccines safety has then been improved through molecular biology techniques, by eliminating virulence genes from the viral recombinant genome (M. A. Liu 2010a; Rollier et al. 2011; Ewer et al. 2016). The most commonly used strategy is to eliminate viral replicative genes, making the viral vector replication-incompetent, to guarantee its safety, without losing antigens expression efficacy. However, replication-competent viruses can still be safely exploited for vaccination, by simply using a lower viral dose or by heterologous prime boost vaccinations in order to reduce innate and pre-existing adaptive anti-viral vectors immunities but mantaining equivalent potency. The success of viral vectored vaccines was primarly reached due to their ability to induce a complete strong immune response, overcoming traditional vaccines limitations. Most of the vaccine approaches are, infact, mainly able to stimulate just a humoral protection, while recombinant

viral vectors arrange other vaccines best features, minimizing the disadvantages (Ewer et al. 2016). In addition to antibody response, being able to enter the cell and express their own recombinant genome, viral vectors are also able to stimulate strong memory and cellular-mediated responses, involving both cytotoxic T lymphocytes (CTL), essential for pathogens and cancer cells clearance, and $CD4^+$ and $CD8^+$ T lymphocytes, secreting different cytokines types and regulating several immune pathways, including intracellular pathogens killing (M. A. Liu 2010a; Rollier et al. 2011; Ewer et al. 2016). For example, the intramuscolar injection of a recombinant adenovirus vaccine can induce transgene expression within 24 hours and trigger the innate immune responses via interaction with Pathogen Recognition Receptors (PRRs) on APCs and macrophages. Conseguentially, APCs will migrate to draining lymph nodes to prime $CD8^+$, $CD4^+$ and B cells, inducing the antigen-specific adaptive immunity. APCs (*Fig.12*) (Ewer et al. 2016).

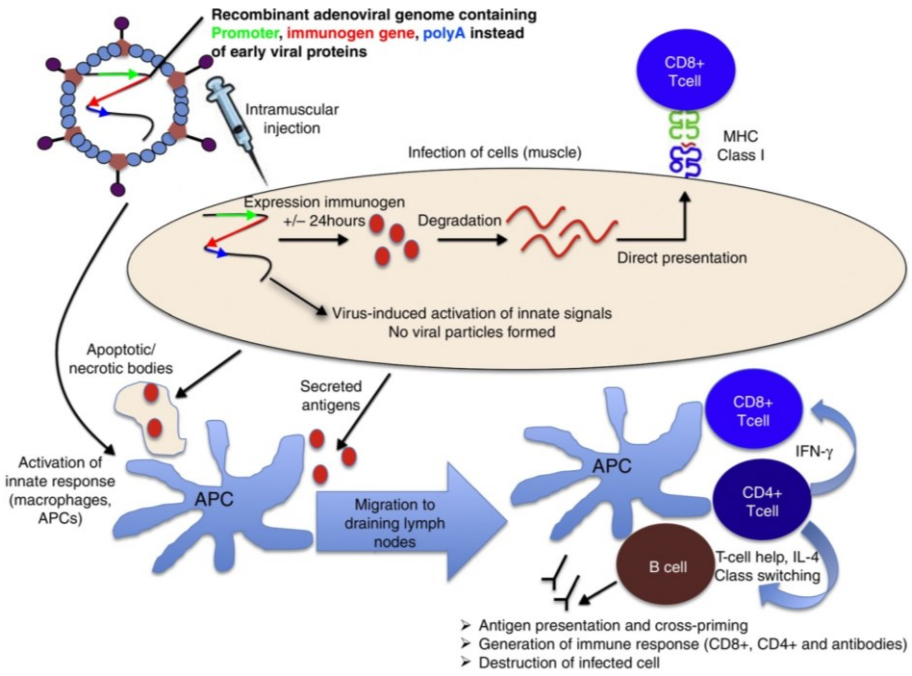

***Figure 12*. Simplified schematic representation of the induction mechanism of transgene-specific humoral and cell-mediated immunities by replication defective viral vector vaccines.** Adapted from (Ewer et al. 2016).

The application of viral-based vaccines in human and veterinary medicine must meet certain safety rules setted by the World Health Organization (WHO). In 2003 and 2013, the WHO has discussed the quality and safety aspects of vaccines based on live viral vectors and

among several issues faced by the Viral Vector Vaccines Safety Working Group (V3SWG) of the Brighton Collaboration (Chen et al. 2015), and has arised the concern of a potential recombination between the live viral vector vaccine and the circulating wild type virus giving birth to a even more pathogenic viral strain. The resulting report has listed the guidelines that a safe viral vectored vaccine must satisfy in order to prove stability of the inserted transgene:

- to verify transgenic sequence presence through PCR;
- to verify transgenic protein expression through Western immunoblotting;
- to verify transgene stability through *In vitro* and *In vivo* passaging;
- to verify recombinant virus non pathogenic phenotiype in order to exclude recombination between vaccine recipient and wild type virus.

(https://www.who.int/immunization/research/meetings_workshops/Oct2013_viral_vector_meeting_comments.pdf) (World Health Organization. WHO informal consultation: development of viral vectored vaccines for HIV, malaria, tubercolosis and other indications: 2013.) (Condit et al. 2016).

The continuous advancement of molecular biology technologies makes it possible to improve viral genome manipulation in order to generate increasingly safer and more adequate viral vectors. Viral vectored vaccine represent somehow a "return back to the origins", to conventional vaccines, as they are made of attenuated or replicative deficient live viruses, but with greater advantages and effectiveness. However, the challenge is to predict which viral vector "type" would make the most suitable and effective vaccine against specific diseases and animal species (*Fig.13*) (Brun et al. 2008).

Delivery system	Advantages	Disadvantages
Modified live vaccines (MLV)	Strong cellular and humoral immune responses; lifelong protection	Reversion to virulence; teratogenicity in pregnant animals
DNA viral vectors	Large capacity for extra genes; multivalent vaccines	
Poxvirus	Replicative poxvirus: cytoplasm replication; long-lasting immunity after single injection; activation of cell and humoral immune responses; easy to produce; low cost; thermo resistance; immunogenic by various routes Non-replicative poxvirus: limited spread; low immunity against the vector; priming young animals with maternal Abs; boost the IR after vaccination; require high doses; DIVA	Immunity against the vector after repeated use (pre-existing immunity)
Herpesvirus	Large genome size; availability of methods to manipulate the genome	Reversion to virulence; integration in the host genome
Adenovirus	Potent humoral and cell-mediated immune responses; intrinsic adjuvant properties, long-term low level antigen presentation; effective oral vaccines	Potential release as GMOs Limited capacity of adenovirus for insertion of foreign genetic material
Baculovirus	Insertion of large foreing DNA fragments; transduction of many cell lines and primary cells; do not replicate in vertebrate cells; no cpe in transduced cells; adjuvant properties	Virus inactivated by complement proteins; few data/studies on immunization
RNA(−) viral vectors	RNA not infectious; stability after serial passages	
Paramyxo	Good humoral and cellular immune induction; complete and lifelong protection without virus shedding, low cost vaccination procedures; chimeric marker vaccines possible	GMO release
Rhabdovirus	Genetic stability; high level expression of foreing proteins; highly immunogenic (humoral, cellular and innate effector mechanisms activated); complete absence of preexisting immunity in animals/humans	GMO release
RNA(+) viral vectors		
Alphavirus	Inductors of mucosal immunity Target dendritic cells (E2); replicons allow high level expression	GMO release
Coronavirus	High capacity among RNA vectors (Coronavirus); secretory immune response; modifiable tropism; non-pathogenic infect most species of interest	Preexisting immunity against the vector
Retrovirus	Good inducers of protective CTL responses; transduction of dendritic cells; genetic stability; long-lasting expression of antigens;	Integration in host's genome
Flaviviruses	Possible interchanging of genes, generating chimeras	GMO release

Figure 13. **Schematic table summarizing advantages and disadvantages of different viral vectors as antigen delivery systems.** Adapted from (Brun et al. 2008)

Viral-based vaccination, particularly focused on large DNA viral vectors, such as poxvirus, adenoviruses and herpesviruses, has been widely and successfully applied in veterinary medicine as a tool to prevent and control infectious diseases spread in livestocks and non-endemic regions (Brun et al. 2008; Draper e Heeney 2010; .Baron, Iqbal, e Nair 2018a; Francis 2018).

- Poxviruses possess large genomes, between 130 and 330 Kbp, that can be manipulated in order to accommodate up to 25 Kbp of single or multiple transgenes (Merchlinsky e Moss 1992), thus functioning as a multivalent vaccine platform (Perkus et al. 1985). The replicative cycle occurs in the cytoplasm of the infected cell, without integration into the host genome, avoiding insertional mutagenesis risk. Poxvirus-based vaccines can be made of recombinant live attenuated poxviruses or either replication deficient ones; the first approach exploits the capability of the attenuated poxviruses to complete their replicative cycle into permissive hosts cells to induce long lasting immunty by stimulating both antibody and cell mediated responses, right after the first administration.

Live attenuated Vaccinia virus, expressing rabies virus surface glycoprotein, has been the first poxvirus to be used as gene delivery vector for rabies eradication in West Europe (Mackett, Smith, e Moss 1982). Various Poxviruses, particularly capripoxviruses, have also been attenuated in order to be used as recombinant vectored vaccines. Compared to attenuated poxviruses, recombinant replicative deficient ones are safer and can be administered multiple times since they induce low innate immunity against the viral vector. On the other side, replicative deficient poxvirus-based vaccines need high titers multiple somministration in order to stimulate immune protection. However, both vaccine approaches can be used as DIVA vaccines, expressing one or more pathogenic antigens (Brun et al. 2008).

- Recombinant adenoviruses (Ad) represent very attractive backbones for viral vectored vaccines, as they are capable to induce a very strong adaptive immune response against the expressed antigens into inoculated mammalian hosts. Although they have been isolated from several different mammalian species, human adenoviruses have been the most studied as gene delivery systems, and particularly Ad serotype 5 (Ad5) has been the most tested for vaccination trials. Both attenuated adenoviruses (Ad Rplus), capable of replicating within permissive host cells, and replicative deficient adenoviruses (Ad Rminus) have been tested, demonstrating that Ad Rplus are more effective as they require lower doses of administration and are also able to stimulate mucosal immunity. However, as Ad Rplus can easily spread in the environment and among other animal species, only Ad Rplus strains, whose innocuity has been officially confirmed, can be applied in the field. Experiments on mice models allowed the studies of Ad5 vectors intrinsic adjuvant features, able to elicit strong innate immunity through induction of proinflammatory cytokines secretion and dendritic cells maturation. In turn, cytokines and dendritic cells seems to elicit a strong adaptive immune response through activation of antigen specific $CD8^{+}$T cells. Human strains of Ad vectors have been extensively tested on mice and non -human primate models first, then extended to animal species of veterinary medicine interest, where to date, some Ad based vaccines allowed the successful eradication of endemic infectious diseases (Brun et al. 2008).
- Herpesviruses possess a dsDNA genome of large size, capable of accomodating variable size transgenes. The possibility to manipulate their genome in order to eliminate virulence genes without preventing the *in vitro* or *in vivo* viral replication cycle is an important feature that makes them excellent vaccine vector candidates. Except Bovine herpesvirus type 4 (BoHV-4), most of the herpesviruses that have been engineered for

vaccination uses belong to the alphaeherpesviridae family. Bovine herpesvirus type 1 (BoHV-1), in 1991, has been the first farm animal alphaherpesvirus to be assessed as heterologous antigens delivery system, using a virion surface display approach (M. Kit et al. 1991; S. Kit et al. 1991). Although this approach has proved to stimulate a highly specific humoral response against the displayed antigens, subsequent BoHV-1-based vaccines have been developed through viral genome modifications in order to insert expression cassettes coding for heterologous antigens. Numerous virulence genes of the herpetic genome, such as thymidine kinase (TK), have been identified for expression cassettes insertion, whose depletion doesn't have consequences on viral replicative process, as previously well described by Donofrio et al. for BoHV-4-ΔTK (Gaetano Donofrio, Franceschi, Capocefalo, Cavirani, et al. 2009). Manipulation of herpetic genomes can be achieved through classical homologous recombination process into eukaryotic cells or more easily through the highly efficient BAC recombineering system into *E. coli* strains (Brun et al. 2008).

Veterinary vaccines

In the veterinary field, vaccines have immediately found ample margin of employment, replacing, for examplethe use of antibiotics and chemotherapy. Vaccines mechanism of action is in fact based on host's natural immune system stimulation, allowing safe consumption of toxic drugs residues free farmed food (P. P. Pastoret 1999). Efficacy and safety of authorized and marketed vaccines are legally guaranteed by 91/412 /CEE and 92/18 /CEE directives, which control and regulate medicinals and veterinary immunized products respectively, and by European guidelines which regulate vaccines production and control. Moreover, animal vaccination represents an extremely importan "tool" for farms infectious diseases prevention and potentially exploitable for the eradication of endemic diseases («Il Progresso Veterinario - Organo Ufficiale della Federazione Nazionale degli Ordini Veterinari Italiani» s.d.). Most of the licensed veterinary vaccines still used today, consist of conventional vaccines, developed decades ago (.Baron, Iqbal, e Nair 2018b). Biotechnological advances have allowed efficient clinical improvement and increased safety of veterinary vaccines (Francis 2018). Biotechnological products seems, in fact, to be safer than traditional ones, and their products consumer, animals and environment safety are guaranteed by 2309/93/CEE, 90/219/CEE and 90/220/CEE directives. However, despite

biomolecular advances, the attual challenge remains to elaborate vaccines ideally capable of maximizing efficacy alongside safety («Il Progresso Veterinario - Organo Ufficiale della Federazione Nazionale degli Ordini Veterinari Italiani» s.d.). Compared to human vaccines, the generation and use of veterinary vaccines require some considerations:

- Costs. Vaccination costs depend on biological and immunological needs of the different livestock animal specie. For example, the need of several vaccine boosts would have a significant impact on costs increasing (.Baron, Iqbal, e Nair 2018a).
- Time. How fast the vaccine is able to induce a complete protective immune response is higly important, expecially during disease outbreacks, in order to prevent the disease spread to other animal species and to the environment.
- Serum-surveillance. Vaccination itself is capable of inducing immunity against a pathogen and prevent its infection but it does not allow the eradication of its circulation (van Oirschot et al. 1986). In case of countries suffering from endemic diseases, in order to implement effective eradication programs, "special" vaccination strategies are needed to allow serum surveillance and being able to distinguish between animals protected by vaccination from animals protected as survivors from wild type pathogen infections (Brun et al. 2008). Marker vaccines, also called DIVA (Differentiation of Infected from Vaccinated Animals) vaccines allow this distinction and makes it potentially possible to generate culling eradication programs for endemic diseases.

DIVA vaccines

DIVA vaccines strategy is based on the absence of one or more pathogen antigenic markers in order to discriminate vaccinated from infected animals based on the presence/ absence of specific detected antibodies. DIVA vaccine formulations usually contain infectious microorganisms with genomic deletions (commonly of surface glycoproteins coding genes) or only viral peptide subunits. gE-deleted Aujeszky's disease vaccine (Pensaert et al. 2004) and IBR-gE deleted infectious rhinotracheitis (IBR) vaccine (Colitti et al. 2018) are examples of veterinary marker vaccines containing genomic deletions available on the market. Genetic modifications can also be exploited to attenuate viral infectivity, for example by deleting thymidine kinase (TK) gene, involved into herpesviral genome synthesis of Aujeszky disease virus, in orded to achieve viral strain attenuation and marker

generation all in one vaccine formulation. Recombinant E2-subunit and recombinant gD-subunit vaccines are, instead, examples of recombinant subunit marker vaccines available on the market against, respectively, classical swine fever (CSF) (Postel et al. 2018) and IBR diseases. These available vaccines have been, for example, exploited for IBR control and eradication in the Netherlands and for the CSF erandication program in Mexico. In order to be materially employable, DIVA vaccines disease controlling and culling programs need high specificity and sensitivity diagnostic assay kits capable of detecting antibodies against these markers («Il Progresso Veterinario - Organo Ufficiale della Federazione Nazionale degli Ordini Veterinari Italiani» s.d.). Immunoassays capable of distinguishing between vaccination induced antibodies and wild type pathogen induced ones, allow the control and validation of DIVA vaccines efficacy (Brun et al. 2008; Blodörn et al. 2014; van Oirschot et al. 1986). To date, the first documented experimental immunoassay for vaccine serum surveillance has been developed by Van Oirschot et al., in 1986, to eradicate from endemic Aujeszky's disease (van Oirschot et al. 1986). Subunit marker vaccines based on non-pathogenic replicating vectors combine the immunogenic efficiency and safety of the non-pathogenic vector with the disease eradication objective of a culling program. The anti-PPR disease recombinant BoHV-4-PPRV-H-ΔTK-based vaccine generatad in this PhD thesis pilot study has shown how the great results, in term of specific anti-HPPR humoral and cellular mediated immune responses, experimentally obtained in mice could possibly be exploited for an efficient PPR eradication program on goats and sheep in affected underdeveloped countries (Macchi et al. 2018).

PESTE DES PETITS RUMINANTS

Peste des Petits Ruminants (PPR) is an underdeveloped countries viral endemic disease, most commonly known worldwide with the “PPR” French acronym, and also other different minor titles, such as "ovine rinderpest", "plague of small ruminants", "goat plague", “syndrome of stomatitis-pneumoenteritis” or "Kata". This disease is caused by a morbillivirus, the Peste des Petits Ruminants Virus (PPRV), a negative single stranded RNA virus belonging to the *Paramyxoviridae* family, *Morbillivirus* genus. As the PPR name says, the disease targets are small ruminants, primarily both wild and domestic, goats and sheep, thus being concerning for small ruminant livestocks-based economy countries, threatening food security and farmers sustainable livelihood across Africa, Middle East and Asia (Parida et al. 2015a). In addition to goats and sheep, other small wild ruminants susceptible to PPRV infection have been identified, such as representatives of the *Gazellinae*, *Tragelaphinae*, and *Caprinae* subfamilies, showing serious illness and mortality. However, it has to be determined whether wildlife can be a PPRV reservoir, as well as they are for Rinderpest Virus (RPV) (M. D. Baron et al. 2016). Goats are generally more susceptible to the infection compared to sheep, which present a higher recovery rate (Parida et al. 2015a). However, after an outbreack, viral variable seroprevalence between sheep and goats have been observed. These facts might be due to many factors, such as livestock managment, viral strains virulence, and hosts density, species and breed (M. D. Baron et al. 2016). For example, Sahelian goats are more resistant to the infection if compared to Guinean dwarf goats, and Alpine goats show high sensitivity after experimental infections with a PPRV Moroccan strain (Hammouchi et al. 2012).

PPRV successfull infections can be simply established by close contacts between animals and it leads to both high mortality and morbidity, up to 90% and almost 100%, respectively. The disease incubation range takes from 2 days to 1 week and it is characterized by fever, nasal-ocular mucopurulent discharges, diarrhea, leukopenia, dyspnea and nasal-oral mucosa epithelium sloughing, due to tongue and gums vesicular lesions. Affected animals usually die within 4–6 days after fever onset. Several secondary pathogens, such as *Escherichia coli*, *Pausterella* and *Mycoplasma spp.*, might also complicate PPR clinical manifestations (Kumar et al. 2014a). PPRV infection leads profound immune suppression, but surviving animals usually develop life-lasting protective immunity and, to date, no asymptomatic healthy carriers have been identified. However, it has been observed that mild disease-affected animals, can allow virus circulation, leading to PPR outbreacks. Seasons and host

factors, such as race, sex and age, seem to influence the disease development, while temperature represents a stringent factor for PPRV infection, since dry environments seems to readily induce virus inactivation (Parida et al. 2015a).

The disease identification initially took a while, as its etiological agent is a Morbillivirus very closely related, and cause similar clinical signs, to the already known Rinderpest Virus (RPV), which is the causative agent of the already globally eradicated rinderpest disease, in 2011 (Kumar et al. 2014b). In addition, PPR clinical signs are very similar and easily confused with pasteurellosis (Taylor et al. 2002), bluetongue, capripox, Foot-and-Mouth Disease (FMD), contagious caprine pleuropneumonia and contagious dermatitis diseases (Singh et al. 2009), thus making it hard to identify it as a separate specific disease. PPR has been first observed in 1942, in West Africa, on Ivory Coast goats and sheep, by Gargadennec and Lalanne who described it as a rinderpest-like disease but with some differences, based on the fact that it did not show any transmission from small ruminants to cattle that were in contact with them (L Gargadennec e Lalanne 1942). A recent serological survey conducted in Senegal, in 2015, showed a 80% rate of PPRV seroprevalence in cattle and small ruminants rich territories, with no reported clinical signs for cattle (M. D. Baron et al. 2016). Cattle can be PPRV infected, but they cannot transmiss the infection to other hosts (Kumar et al. 2014b), even if Ali Khan serological analysis evidenced a possible transmission from sheep and goats to cattle, and both domestic and wild African buffaloes (*Syncerus caffer*), highlighting the need of including PPR serology in sheep and goats sero-monitoring programme for a better indication of national herd immunity against PPR disease (Khan et al. 2008). In fact, even if PPRV is not considered pathogenic for cattle and buffaloes, over the 10% of these species exposed to PPRV enzootic regions, may present seroconversion. Moreover, highly fatal PPRV infections have been reported in India, in domestic buffaloes (*Bubalus bubalis*), where it has been possible to experimentally reproduce the disease. In addition, PPR has also been suggested to occur in camelids, even if there have been no successfull attempts of experimentally reproducing the disease. To date, it remain unclear whether PPRV infected buffaloes and camels might be able to transmit the infection to small ruminants (M. D. Baron et al. 2016). Beyond cows, virus transmission among susceptible animals in close contact to each others occurs through nasal, ocular, mouth and anal excretions or exhaled aerosol (Parida et al. 2015a).

Only in 1956, proving that PPRV and RPV are antigenically and genetically closely related viruses, but distinct from each other (Munir 2010a), PPRV has finally been officially identified as the PPR causative agent (A. Diallo et al. 1989) and more than twenty years

later, in 1979, it has been classified as a Morbillivirus, belonging to the *Paramyxoviridae* family (P. J. Gibbs et al. 1979). PPRV first isolation date back to 1962 from sheep cell culture, while in 1967 it has been observed for the first time with electron microscopy (Kumar et al. 2014a).

To date the *Morbillivirus* genus comprehends five different viruses in addition to RPV and PPRV: Measles Virus (MV), Canine Distemper Virus (CDV), Phocine Distemper Virus (PDV), Cetacean Morbillivirus (CeMV) and Feline Morbillivirus (FMV); where CeMV is further sub-classified into the Porpoise Morbillivirus (PMV), Pilot Whale Morbillivirus (PWMV) and Dolphin Morbillivirus (DMV), three groups (Kumar et al. 2014b), moreover numerous morbilli-like viruses in bats and rodents have been identified (Parida et al. 2015a).

Taxonomy

PPRV is classified as a viral species belonging to *Morbillivirus* genus, *Paramyxovirinae* subfamily, *Paramyxoviridae* family and *Mononegavirales* order, as shown in *Fig.14* (Parida et al. 2015a). Among the *Mononegavirales* order are included some of the most concerning and important viral pathogens for medical and veterinary sciences and PPRV represents one of them.

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

Figure 14. **PPRV Taxonomy. Classification of viruses belonging to the Mononegavirales order**. Adapted from (Parida et al. 2015a)

Morbilliviruses are then generally further classified into different monophyletic clades based on their genome structure and their ability to selectively infect different animal species, as shown in *Fig.15,* where, the schematic phylogenetic tree was constructed using partial sequences, of 230 nt, of Nucleocapsid (N) genes.

For example, Measles virus infection concerns only human and non-human primates, while PPRV and RPV infections are restricted to ruminants. Initially, based on their historical detections and due to their available data scarcity, cetacean morbilliviruses have been classified into Dolphin, Porpoise and Pilot Whale Morbillivirus species, respectively infecting dolphins, porpoise and whales. Further identifications and genetic characterizations of these viruses revisited previous classification, uniting the three species under one single cetacean morbilliviruses monophyletic group. Recently, new cetacean isolates that clusterize within the cetacean morbilliviruses, but are genetically divergent to the ones previously characterised, have been identified, thus extending the complexity of this group (Parida et al. 2015a).

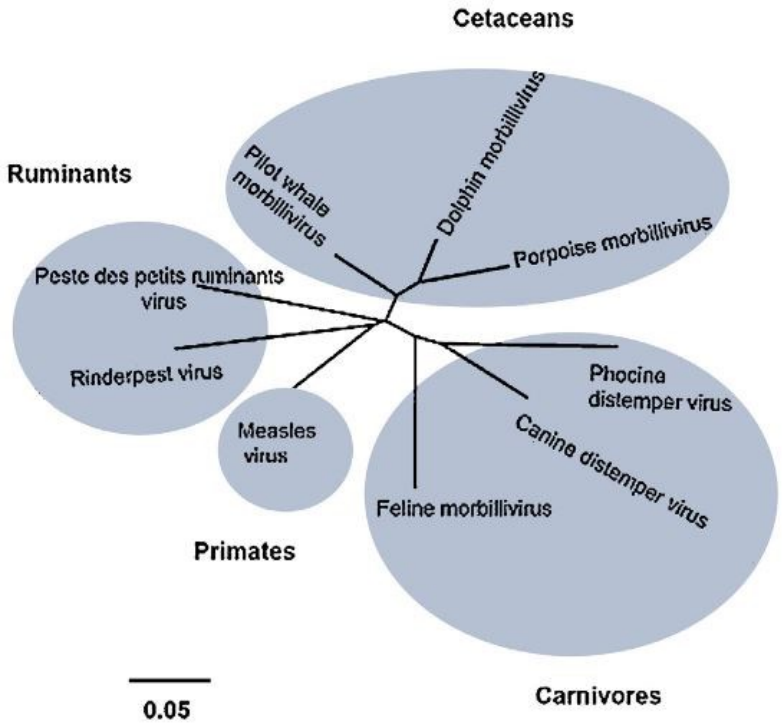

Figure 15. **Phylogenetic tree of different Morbillivirus species.** Adapted from (Parida et al. 2015a).

Morbilliviruses are generally classified as strictly related to their ability of infecting specific animal species, like MV exlusively infects human and non-human primate hosts, RPV infects the members of the Artiodactyla Order and CeMV infection targets are the acquatic mammals. This classification, however, is not totally true realistic and representative for the PPRV, whose infection does not seem to be restricted only to small ruminants, as there have been cases where it has been reported as the causative agent of respiratory diseases with high mortality also in african camels (Roger et al. 2000; 2001), in an asiatic lion, even if only in a single occasion (Balamurugan, Sen, Venkatesan, Bhanot, et al. 2012) and few outbreacks have been reported from indian buffaloes (Govindarajan et al. 1997), cattle (J. Anderson e McKay 1994; Lembo et al. 2013; A. Sen et al. 2014), and pigs (Nawathe e Taylor 1979), showing just a subclinical infection without viral excretion, thus not considered concerning for PPRV epidemiology. Moreover, several PPRV-infected wildlife species, mainly living in semi-free conditions, have also been reported, but their epidemiological role remain unclear. However, PPRV preferential hosts remain both wild and domestic goats and sheep. CDV seems to be the most promiscuous morbillivirus, since it was initially thought restricted to canids infections, but has then been observed in other different animal species, such as lions, tigers, hyenas, polar bears and non-human primates. Morbilliviruses are then extensively, better characterized based on MV, RPV and CDV molecular data. MV and, to some extent, RPV and CDV, represent morbilliviruses prototypes, while, in comparison, PPRV is not yet as well characterized. However, it is well known that viruses among Morbillivirus genus are well conserved, with different morbillivirus species sharing similar characteristics (Parida et al. 2015a).

Epidemiology and transmission

PPR first official report as a distinct disease dates back to 1942, to Gargadennec and Lalannene observations of a rinderpest-like disease afflicting goats in Côte d'Ivoire in 1940. They identified the so observed disease distinguishing characteristic, of not being able to be transferred from affected small ruminants to cattle that were in contact with them, and named it as "plague of small ruminants". For some times, PPR reports have been confined to West Africa, and not long after, a similar disease has been reported in Benin, where it has been named as "peste des espèces ovine et caprine", meaning: plague of sheep and goat species (M. D. Baron et al. 2016). PPR could then have been recognized in several others occasion all across Africa, but as it was most likely considered as a rinderpest-like disease, no further

investigations have occurred. Only in the eighties, it has finally been considered as a different pathology from rinderpest, caused by a distinct etiological agent (Parida et al. 2015a). In 1984 Taylor (Taylor 1984) pointed out that the outbreacks erroneously recognized as rinderpest-like, that occurred in 1871 in Senegal, and in 1927 in French Guinea, may have in fact been PPR, and that the disease outbreack in Sudan, initially reported as rinderpest, ten years later came out to have also been PPR (M. D. Baron et al. 2016). PPRV recognized geographical distribution has then increasingly expanded across the northern, central, and near Est African countries as well as West, central and South Asia, where it has been first reported in India in 1989 (Shaila et al. 1989), and in China by the end of 2009 (Parida et al. 2015a; M. D. Baron et al. 2016).

The livestock movements across the African and Asian countries and regions might have facilitated PPRV expansion, but its apparently increased geographical distribution must have been mainly due to PPRV specific diagnostic tests development and availability, but also to successful rinderpest global eradication. Rinderpest disease may only occasionally affect small ruminants, but since its clinical signs, except for the respiratory syndrome which is a typical for PPR acute form, are very similar to PPR, it has always been considered the diagnosis of choice, at least until RPV have gradually been globally eradicated. Moreover, as RPV goats and sheep subclinical infections protect against PPRV, RPV might also have had a direct effect on PPRV epidemiology by limiting its spread to the countries where rinderpest was endemic. PPRV wide spread might have begun when rinderpest eradication came under control (M. D. Baron et al. 2016). Comparative molecular epidemiology analysis between small sequences of the F (322nt), H (298nt) or N (255nt) PPRV genes, defined the existance of four different viral lineages, referred as I-IV. Depending on whether F or N genes are being considered, lineages I and II nomenclature are differently referred: lineage II based on F data is referred as lineage I if based on N data and vice versa. However, recent and more in-depth comparative analysis between F, H and N partial sequences showed higher conservability for H and F sequences compared to N, much more variable. Based on these data the novel PPRV isolates are more selectively and precisely classified based on N gene region. Even if N gene is more suitable for phylogenetic distinctions among closely related circulating PPRVs, Liang et al. (Liang et al. 2016) have studied PPRV epidemiology based on the highly conserved H gene, showing a highly similar evolutionary relationship among the four lineages (*Fig.16*).

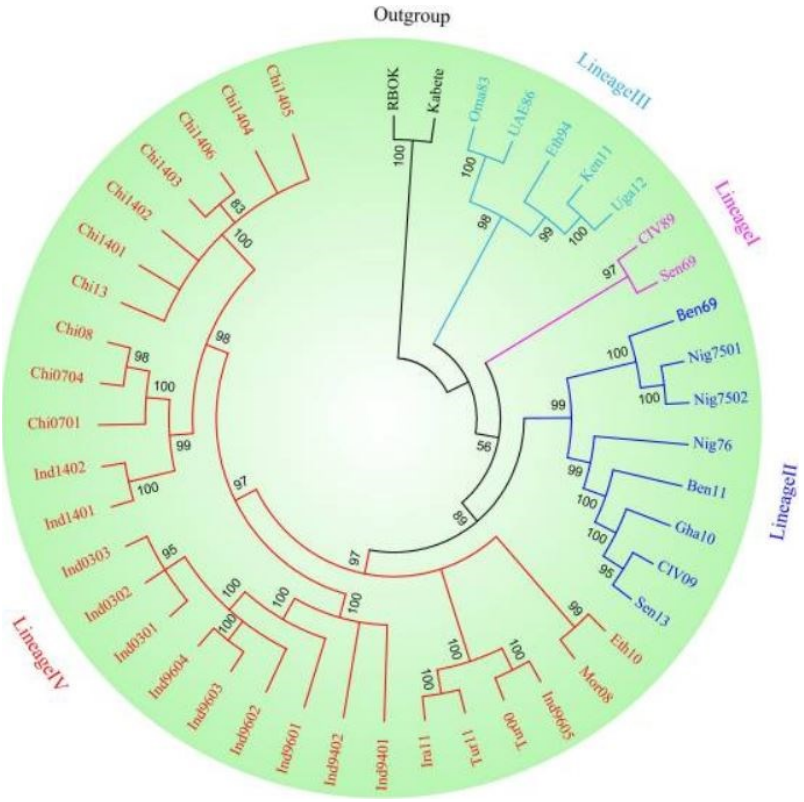

***Figure 16*. PPRV phylogenetic tree, based on the Hemagglutinin (H) gene.** Adapted from (Liang et al. 2016)

The phylogenetic tree has been constructed with the Bayesian method GTR+I+G model. Topology patterns show all four lineages strains. Analysis has been performed with TOPALi v2.5 package.

As shown in *Fig.17*, lineages I-III have historically been found in Africa and referred in accordance with the PPRV apparent spread from West Africa, where lineage I belongs to Senegal and Ivory Coast and lineage II to Ghana and Nigeria, all the way to East Africa, where lineage III has been identified. Lineage IV, instead, was mainly found in Asia and Middle East, including isolates from India, China, Ethiopia, Morocco, Turkey, and Kurdistan. There have also been few lineage III exceptions being reported in Yemen and Oman, and some hybrid lineages, deriving from lineages III and IV, reported in United Arab Emirates and Qatar. Recently, lineage IV has moved from Asia and reached all PPR endemic territories, replacing the other three lineages in African outbreacks. Its apparent expansion is corroborated by a constant increased disease incidence all across Africa, suggestin also a PPRV virulence increasing (Parida et al. 2015a).

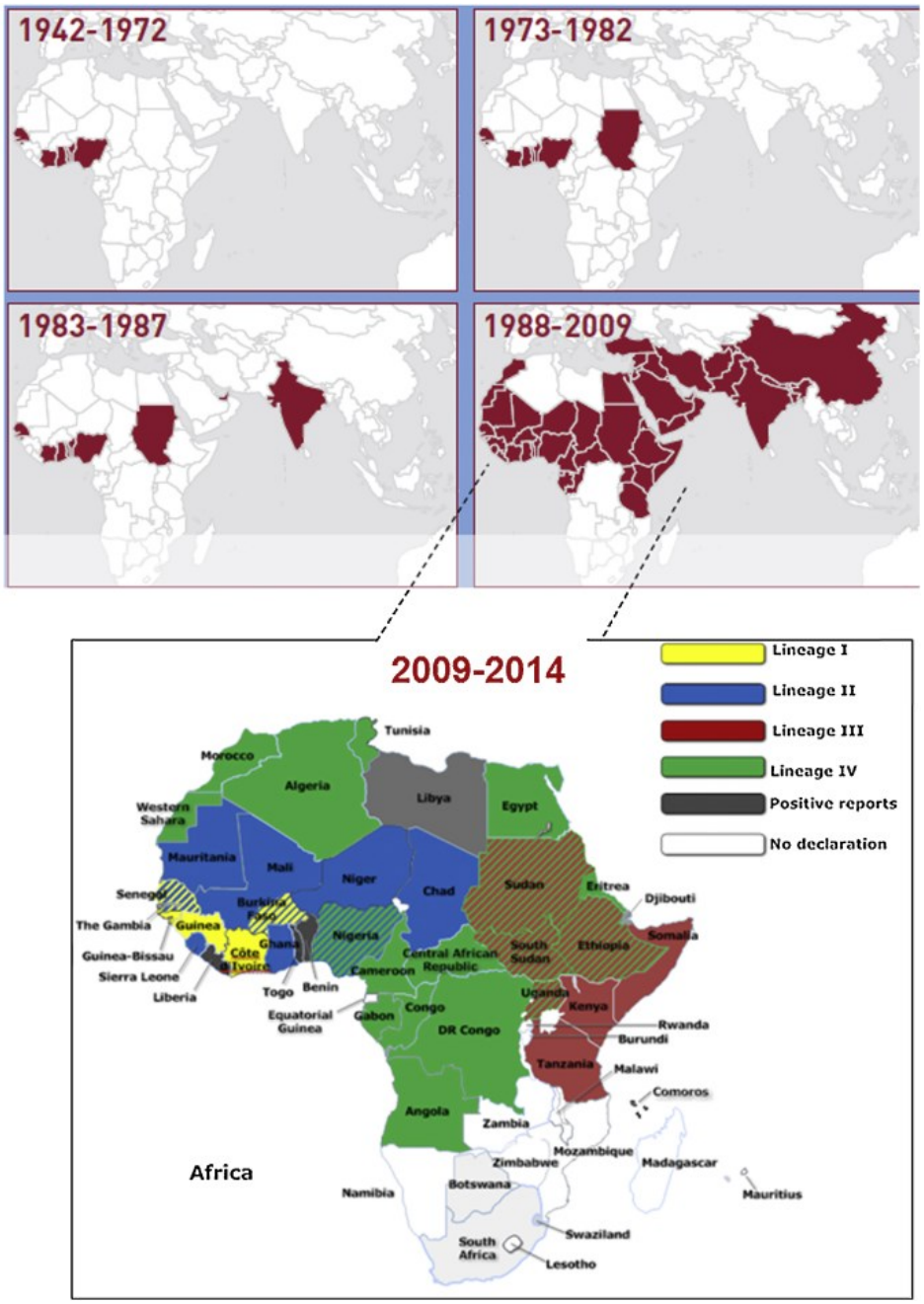

***Figure 17*. PPRV lineages global spread since its first identification in 1942, to 2014, with in-depth analysis of its recent distribution in the African continent.** Adapted from (Parida et al. 2015a)

Recently, PPR disease has dramatically continued its African expansion to Southern territories, including Tanzania, Gabon, Somalia, Kenya and the Democratic Republic of Congo. In October 2012 and July 2015 PPRV infections were first reported in Angola and Zambia, respectively, to the OIE. These field and laboratory data increase the concerning of PPR potential spread to sheep and goats- rich neighboring countries, like the Republic of South Africa and Mozambique. PPRV African expansion has concerned also the Northern states. Even if the infection was already present in Egypt since 1989 and in Tunisia since 2006, the first officially reported PPRV identification in North Africa has been in Marocco, in 2008, where it then reemerged in 2015, after a previous mass vaccination campaign that seemed to have eradicated it. Disease relapse might have been caused by intense sheep and goats movements as well as illegal cross-border trades, thus national control strategies for a definitive PPRV eradication is highly needed. Moreover, since 2011, PPRV has now also been identified in Algeria. In addition, its spreaded distribution have reached the European and Asian countries. Twelve outbreacks have been reported in Turkey during 2011-2012, and in Asia, where after the first identification in Tibet, in 2007, PPR outbreacks have recently been reported in China and Kazakhstan during 2013-2015. Interestingly, the circulating viruses were genetically much closer to the ones that have been identified in Pakistan or Tajikistan than to those that have been isolated in Tibet. Although PPRV geographic origin is not clear, recent Bayesian molecular studies on PPRV ancestor origin and its relation with the other morbilliviruses, estimated that the most recent common ancestor among the four lineages might date back to the beginning of the 20th century, before PPRV first recorded description in 1942. Lineage III has been proposed to have been the first to have diverged and for each individual lineages the ancestral virus location have been determined. Nigeria, Senegal, Nigeria/Ghana, Sudan and India have been identified as ancestors arise locations for PPRV, lineages I, II, III and IV, respectively. Moreover, Bayesian phylogenetic analysis have highlighted that RPV is much more closely related to MV, than PPRV (Parida et al. 2015a; M. D. Baron et al. 2016). However, based on its rapid expansion during the last two decades, PPR potentially represents the current most economically important disease affecting developing territories, where domestic goats and sheep play a key role in sustainable agriculture and development (M. D. Baron et al. 2016). Because of its alarming spread, the Food and Agriculture Organization of the United Nations (FAO) and the International Organization Epizootic (OIE), have now focused their efforts into PPR control and eradication (Mantip, Shamaki, e Farougou 2019).

Transmission

Small ruminants, and expecially domestic ones, represent PPRV targets and its only contagious source. PPRV particles excretion in the exhaled air is maximum during the early infection stages, thus, as observed for RPV, potentially allowing its non-contact transmission. Infected animals excrete the virus through feces, saliva and nasal-ocular mucopurulent discharges, containing large amounts of viral antigens. For example, it has been observed that for at least two months after natural infection, goats excrete PPRV RNAs or antigens in the feces, but is not known if infectious particles are present too. Since PPRV is rapidly inactivated in the environment, its most frequent transmission occurs by direct contact between infected and healthy animals. However, national epidemiological controls must not exclude potential indirect viral transmission with contaminated materials. Moreover, since the infection peacks mainly occur during dry, cool seasons of African endemic territories, PPR is commonly considered a seasonal disease. However, its transmission is not restricted to dry periods but can occur all over the year.

PPRV speedly spreads among immunologically naive small ruminants populations, thus it is commonly believed to only persists in large populations of newly susceptible hosts, such as newborn, newly purchased or migrating ruminants.

Statistical estimates collected on fields suggest that, in order to stop PPRV transmission and spread, vaccinations should be able to protect up to the 75-85% of small ruminants populations. However, genetic populations eterogenicity and fields characteristics, such as spaces and time, differently influence PPRV pathogenicity and persistance, and animals susceptibility, thus making it hard to establish a general eradication protocol. Indeed, different breeds coexistence and livestock trades, favor PPRV persistance and its spread to new territories.

Stricter border controls, as well as the presence of adequate protections against PPRV infection, make Europe relatively well shielded to PPR spread risk from Northern Africa. Mass vaccination campaigns should quickly improve China and India PPR situation, while PPRV presence in Eastern Asian territories still remains highly challenging. However, PPR most concerning situation is in Africa, where fourty-eight countries counting over 600 million small ruminants are likely to contract the infection and to introduce it to virus-free countries (M. D. Baron et al. 2016).

Clinical signs

PPR incubation period typically takes 4 to 6 days, but it may also take a wider range from 3 to 14 days. In the initial disease acute stage, infected animals develop high fever, up to 41°C, that might last 3 to 5 days and can be followed by other clinical signs, such as anorexia, depression and muzzle dryness. During disease course, salivation increases, lachrymal and watery nasal discharges gradually become mucopurulent, and oral cavity lesions might get necrotic. In case of severe disease, these necrotic lesions get worse and fibrin deposits appear on the tongue. The disease late stages are characterized by diarrhoea, cough and laboured abdominal breathing. The disease progression then ends up with dyspnea, progressive weight loss and emaciation, leading to the animal death. However, in fewer cases, particularly of mild infections, animals go through convalescence, getting back to a health status within 10 to 15 days. PPR causes a high fatality rate, expecially during its acute form, and a morbidity rate up to 100%.

During the initial stage of the infection, virus excretion is very high in the exhaled air, thus potentially allowing its non-contact transmission over at least few metres, similarly to RPV. A large amount of viral antigens is then also present in saliva, nasal discharges, lachrymation and faeces. In partuclar, goats expell PPRV antigens and RNAs within the faeces for at least two months after the natural infection, but it is not clear whether the expelled virus is infectious or not. Since it is known that PPRV is quickly inactivated in the environment, its transmission mostly occurs by close direct contact between infected and healthy animals, but its indirect transmission might also occurs by contact of recently (few hours) contaminated materials. Both transmission ways must be considered in epidemiological control measures. Since it rapidly spreads in immunologically naïve flocks, it is commonly believed that PPRV might only persist in large animal populations in which new susceptible hosts, such as newborn, purchased or migrating animals, are present (Mantip, Shamaki, e Farougou 2019).

Morphology

Thanks to electron microscopy, morbilliviruses general morphological structures have been studied and described as enveloped and pleomorphic particles of about 400-500 nm in size. Peste des Petits Ruminants virions report morbilliviruses typical characteristics, as shown by *Fig.18* electron microscopy (E. P. Gibbs et al. 1979; Parida et al. 2015b).

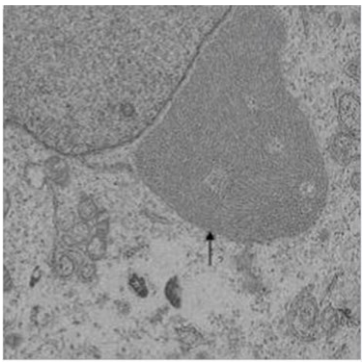

Figure 18. **Electron microscopy detail of PPRV nucleocapsid assemblation within a host infected cell cytoplasm during the viral replication cycle.** Adapted from (Parida et al. 2015b)
The arrow indicates the viral RNA genome encapsulated together with the viral Nucleocapsid proteins (N).

During virion budding through the host infected cell membrane, PPR virions outer envelope acquire the viral surface glycoproteins, Fusion peptide (F) and Hemagglutinin glycoprotein (H), within its thickness. The viral envelope inner surface is delimited by the Matrix proteins (M), which, in turn, delimits a linear, single stranded, negative sense RNA genome encapsidated into a helic shaped Ribonucleoprotein (RNP) complex structured by the combination of the Nucleocapsid protein (N), the Phosphoprotein (P) and the RNA dependent RNA Large polymerase (L) (*Fig.19*) (Parida et al. 2015b).

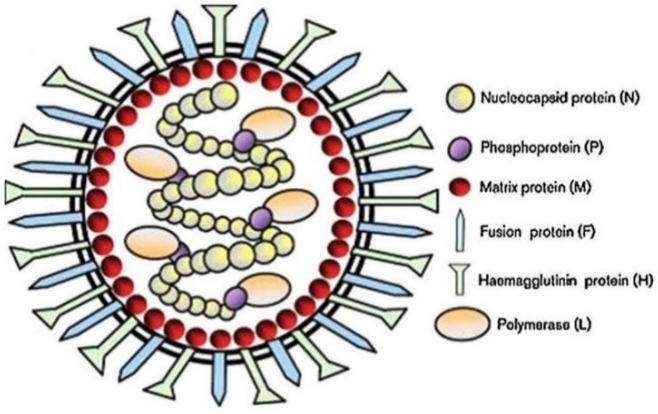

Figure 19**. Schematic representation of PPR virion structure and its structural proteins.** Adapted from (Parida et al. 2015b)
Legend: Nucleocapsid (N), Phosphoprotein (P), Matrix protein (M), Fusion peptide (F), Hemagglutinin (H), RNA dependent RNA Large polymerase (L).

Genome structure

PPRV genome is a non-segmented, single stranded, negative sense RNA molecule of 15,948 nucleotides in length (*Fig.20*). However, a PPRV genome variant with six additional nucleotides has been recently detected during the Chinese epizootic (M. D. Baron et al. 2016). The genome contains six transcription units: N, P(V/C), M, F, H, L from 3' to 5', encoding for eight total proteins, six of which, the Nucleocapsid (N), the Phospho (P), the Matrix (M), the Fusion (F), the Hemagglutinin (H), and the Large (L) proteins, are structural components while the V and C proteins are non-structural ones. The C protein is generated through the use of a different start codon of the P gene open reading frame while the V protein is generated through RNA editing of P mRNA (Kumar et al. 2014b).

Multiple N protein copies encapsidate the PPRV genome and protect it from nuclease activities, while several P and L proteins copies are included within the nucleocapsid since they are indispensable to start the mRNA transcription from the viral genome (M. D. Baron et al. 2016). Between each gene there is a GAA conserved triplette, named Intergenic Region (IG), which is also present at the left side of the first N gene sequence and at the right side of the last L transcriptional unit. IGs are all well conserved among PPRVs genomes, except for the last 5' IG where the GAA triplette is substitued by a GAU one. PPRV genome is flanked by a 3' Genome Promoter (GP) and a 5' Antigenomic Promoter (AGP) regions, which are highly conserved among morbilliviruses since they are involved into viral replication and propagation pathways. The 3' GP sequence includes the 52 nt leader region together with the 3' N gene Untraslated Region (UTR) and the first 3' genomic IG, while the 5' AGP sequence includes the 37 nt trailer region together with the 5' L gene UTR and the last 5' genomic IG (Kumar et al. 2014a; Parida et al. 2015b). Morbilliviruses UTRs are particularly abundant at the 5'-M and 3'-F ORFs, and do not seem to affect the *in vitro* viral replication (Liermann et al. 1998); however several studies demonstrated that morbilliviruses non-coding RNAs are involved in determing viral virulence (D. E. Anderson et al. 2012) through host immune system modulation (R.-Y. Chang et al. 2013; Samanta e Takada 2010), plaques generation (Esteban et al. 2014) and latent infection establishment (wu et al. 2011).

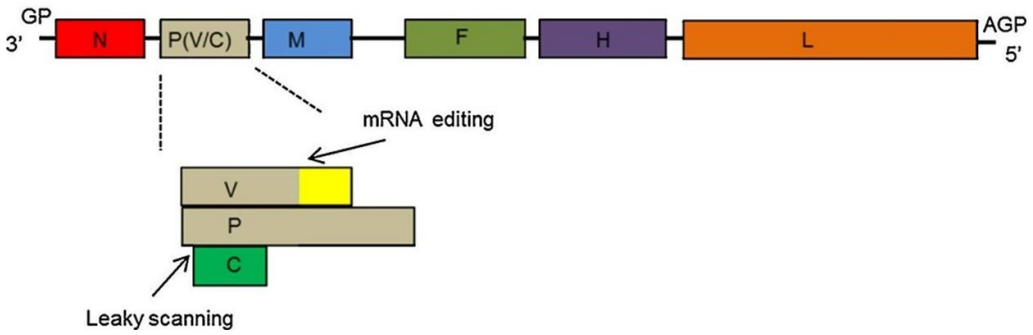

Figure 20. **Schematic representation of PPRV RNA genome**. Adapted from (Parida et al. 2015b) PPRV genome is composed of six transcriptional units N, P, M, F, H and L encoding for structural proteins. Leaky scanning and mRNA editing of P gene generate C and V non structural proteins, respectevely.

PPRV proteins

Nucleocapsid protein, N. Nucleocapsid protein N is a ~58 KDa structural protein, in charge of fully encapsidate into nucleocapsid like aggregates the viral genome, so that is never found as nacked RNA into the cytoplasm of the host infected cell (Ismail et al. 1995; Kumar et al. 2014a), and thus faciliting genome transcription and replication (Rahaman et al. 2003). N protein is quite conserved sharing 67-74% of identity, among morbilliviruses and it represents the most produced protein in high amounts.

Phosphoprotein, P. Like all other morbilliviruses, PPRV produces the phosphoprotein (P) itself, forming the functional replication complex together with the Nucleocapsid protein (N) and the RNA dependent RNA large polymerase (L) proteins and interacting with the appearently multifunctional C and V non structural proteins (M. D. Baron et al. 2016). Specifically, PPRV P has a predicted molecular weight of ~60 KDa but it has been shown to migrate at ~80 KDa in SDS PAGE assay, probably due to the phosphorilation process occurring at its Serine and Threonine residues. The Serine residue located in position 151 represents the protein most likely phosphorilation site as it is highly conserved among morbilliviruses, even if P protein appears to be the least conserved protein among morbilliviruses. P protein is a costitutive component of the RNP complex acting as a co-factor of the L protein. P active form is a tetramer and the interaction established with N proteins into RNP complex is “weak” enough to allow polymerase L progression on the RNA template (Kumar et al. 2014a).

Matrix protein, M. Matrix protein M has a ~38 KDa molecular weight (F. Liu et al. 2013; Kumar et al. 2014a) and shares an almost 91% of aminoacid identity with the other morbilliviruses. This protein seems to play a key role during virions assembly and budding through the host infected cell membrane by targeting F and H glycoproteins to the envelope surface (Kumar et al. 2014b), however, M precise role and position within the the virion is still a matter of research. The most accepted hypothesis suggests its potential role in linking the nucleocapsid to the membrane, even if there are no data proving the existance of specific stable bonds between M and either the N protein or the viral glycoprotein cytoplasmatic domains. This hypothesis is corroborated by recent studies on baculoviruses expressed PPRV proteins, where it has been observed that either M with N, or M with viral glycoproteins, co-expression leads to Virus-Like Particles (VLPs) generation. On the contrary, the block of M protein synthesis in PPRV infected cells leads to viral release reduction and to cell-cell fusion increasing, suggesting that PPRV M protein is actually involved into virus assembly. As it has been previously shown for the analogous MV protein, also M protein might exist as stable structure on PPRV nucleocapsid surface and, in addition, it has also been proposed that M protein spacial distribution changes during virus assembly might regulate PPRV F protein activity (M. D. Baron et al. 2016).

Envelope glycoproteins

PPRV envelope surface is studded by the Hemagglutinin (H) and Fusion (F) glycoproteins, responsible for virion attachment and fusion to the host cell surface, respectively. (M. D. Baron et al. 2016)

Fusion peptide, F. PPRV F glycoprotein is a type I membrane protein higly conserved among morbilliviruses, but characterized by an amino-terminal signal sequence highly variable both in lenght and in aa content (M. D. Baron et al. 2016). The protein has a predicted molecular weight of 59,137 KDa and plays a crucial role during the first steps of viral infection and replication, allowing the fusion between the viral and the host cell membranes and triggering viral nucleocapsid release into the host cell cytoplasm. The other morbilliviruses need both fusion peptide and hemagglutinin/ hemagglutinin neuraminidase surface glycoproteins to complete the fusion process, while PPRV only requires the F protein (Seth e Shaila 2001; Kumar et al. 2014a). Moreover, it has also been obssrved that F protein has also both hemolysis and autophagy activities. In order to perform its function, F must first undergoes

structural modifications whitin the host infected cell cytoplasm. As well as the other paramyxoviruses, PPRV synthetises the F protein as an F0 precurson on the Rough Endoplasmic Reticulum (RER) ribosomes. F0 is subsequently cleaved into F1 and F2 fragments by the furin endopeptidase enzyme inside the host cell Golgi apparatus. The cleavage occurs at the F0 conserved sequence: Arginine-Arginine-X-Y-Arginine, where X could be any aminoacid and Y must be an Arginine or a Lysine. F1 and F2 fragments remain linked together by a disulphide bond and F1 fragment mediates the viral particle anchoring to the host cell membrane. F1 fragment is composed of four conserved motifs:

- The Fusion Peptide (FP), which is positioned at the F1 N-terminus and its function is to anchor itself into the host cell plasma membrane double layer.
- The Heptad Repeat 1 (HR1) and Heptad Repeat 2 (HR2), that, by interacting with each other, approach viral and host cell membranes close together enough to fuse to each other.
- The Transmembrane domain (TM).

In order to perform its fusogenic function, during cytoplasm to plasma membrane translocation, F protein is glycosylated by host cell enzymes in the N-linked conserved sites, Asparagine-X-Serine/Threonine. The glycosilation process seems to be important also for F transportation into host cell plasma membrane (Kumar et al. 2014a). Differently from rinderpest, canine distemper and measles viruses, where it has no effect or even an inhibiting effect, the PPRV F gene 5' UTR sequence, measuring over 500bp in lenght, promotes the protein translation and expression by stabilizing the mRNA in the cell cytoplasm (M. D. Baron et al. 2016).

Hemagglutinin, H. PPRV Hemagglutinin (H) is a 609 aminoacids protein of 68,803 KDa, relatively conserved among different PPRV strains. Specifically, H is a type II glycoprotein, with an N-terminal cytoplasmic tail, a transmembrane domain located inside the viral membrane, a membrane proximal stalk region and a large C-terminal receptor-binding head domain protruding on viral surface. The 35-38 N-terminal aminoacids constitute H hydrofobic domain, that allow protein anchoring into the viral membrane. Differently from the other morbilliviruses where H protein only possesses the heamagglutinin function, PPRV H possesses both hemagglutinin and neuraminidase activities. These functions, together with F fusogenic activity, mediate viral attachment to the host cell receptors, determining viral

adsorption and entry into the host cell cytoplasm, where new PPR viral particles are produced.

Despite the predicted 67 KDa molecular weight, due to glycosylation changes within the host cell, H proteins from PPRV infected cell lysates, show a higher molecular weight of about 70 KDa on SDS PAGE. Like F, also the H protein is synthetised on RER ribosomes and undergoes N-glycosilation through the Golgi apparatus. Glycosilation varies among different morbilliviruses and PPRV strains, affecting H protein antigenicity. Due to the glycosilation state and its functions, H protein manages PPRV pathogenicity, representing its surface immunodominant antigen, thus, the most ideal candidate for vaccines formulations against the peste des petits ruminants disease. It has, in fact, been shown that anti-H neutralizing antibodies are protective against PPRV infection.

As well as all the other morbilliviruses, PPRV exploits the lymphoid CD150 as primary receptor and the cellular Nectin-4 surface receptor on epithelial cells. CD150 is also known as Signaling Lymphocyte Activation Molecules (SLAM), and is present on several lymphoid cells surfaces, such as macrophages, activated T and B lymphocytes, mature dendritic cells, and platelets (Hashiguchi et al. 2011; M. D. Baron et al. 2016), but it is also exploited as a primitive hematopoietic stem cells marker. CD150/SLAM receptor represents SLAM receptors family prototype member, including 2B4, CD48, CD84, CD229, CD319, and Natural killer, T- and B-cell antigens (NTB-As), through which important regulatory signals in immune cells are mediated. All SLAM family receptors are type I membrane proteins containing an extracellular domain with V-set and C2-set immunoglobulin (Ig)-like domains, a transmembrane domain and a cytoplasmic tail. The membrane distal V-set Ig domain itself is already sufficient for the receptor binding activity (Hashiguchi et al. 2011). It has been demonstrated that, during viral attachment, PPRV interacts with SLAM receptors through H glycoprotein that cleaves the receptor carbohydrate portion at the sialic acid residue (Kumar et al. 2014b; M. D. Baron et al. 2016). This cellular receptor can also acts as a self-ligand by interacting with an analogous SLAM receptor on an adjacent cell surface at a low affinity (Kd) of about 200μM, thus its function and distribution on cell surfaces can explain MV tropism and immunosuppressive nature. Despite CD150/SLAM represents morbilliviruses most exploited cellular receptor, also Nectin 4 receptor on polarized epithelial cells surface has been shown to support virus attachment to the host cell (Hashiguchi et al. 2011). Morbilliviruses, infact, initially infect immune system cells by binding the surface CD150/SLAM receptor and then spread over to epithelial cells (X. Zhang et al. 2013). Several studies on PPRV distribution during infection early stages showed that

early in infection the virus is primarily found in the lymph nodes while later on it can be detected in epithelial tissues, apparently via Nectin 4 receptor. Nectin 4 is in fact only located on polar epithelial cells basolateral side, thus PPRV infection on epithelial cells can only occur after viremia establishment (M. D. Baron et al. 2016).

Noyce et al. (Noyce et al. 2011) microarray experiments identified the human cell marker PVRL4 (Nectin 4) as the respiratory tract epithelial cells receptor exploited by several WT MV strains to attach the host cell. Nectin 4 belongs to the Nectin and Nectin-like (Necl) proteins family, including Nectin 1-4 and Necl 1-5. All this family proteins contain three Ig-like domains (V-, C-, and C-sets) and are involved in several cellular functions, such as cell to cell adhesion, cell polarization, survivial, movement, proliferation, differentiation and immune recognition processes. Nectin family proteins *cis/trans* homophilic or heterophilic dimerization seem to play a key role in facilitating all these cellular functions. For example, Nectin 4-Nectin 1 *trans*-homophilic or heterophilic interactions are involved in adherens junction formation (X. Zhang et al. 2013).

Although all morbilliviruses apparently exploit the same cellular receptors well conserved among mammals, morbilliviruses H protein sequences are not as well conserved as F protein sequences. The majority of these sequence differences locate in the H stalk region, responsible for cellular receptor binding by mediating itself oligomerization and F protein interaction. Morbilliviruses H stalk sequence differences might also explain why PPRV H and F proteins efficiently interact to each others but not with RPV ones. Interestingly, Das et al. (Das, Baron, e Barrett 2000) have generated RPV genome recombinant cDNA copies by replacing H or F or both genes with PPRV corresponding ones, trying to improve currently available PPR vaccines. During chimeric viruses reconstituition on Vero cell monolayers, only the chimeric RPV recombinant for both PPRV H and F genes has been recovered, proving that specific functional interactions occur between PPRV H and F proteins (M. D. Baron et al. 2016). Moreover, these H sequence differences also explain why different MV-Hs show variant binding characteristics. Despite both WT and vaccine strain MVs can use the lymphoid SLAM and epithelial Nectin 4 receptors, in addition to those molecules the Edmonston (Ed) vaccine strain of MV also mainly recognize the complement regulatory molecule CD46 (Hashiguchi et al. 2007; X. Zhang et al. 2013).

Since recent reports have observed that MV-SLAM interaction not only represents virus invasion first step to the host, but also the main cause of pathological changes and clinical symptoms manifestation in the host organism, it is of great interest to explore H protein interaction to the SLAM receptor and understand the protein-protein domains/motifs

involved. The identification of morbilliviruses H small antigenic determinants or epitopes inducing neutralizing antibodies generation would indeed greatly help the generation of antiviral drugs targeting the protein specific antigenic targets and increase vaccines efficiency (Liang et al. 2016). To date, only the Ed MV H protein crystallized structure is available and, since there are H protein structural high similarities among morbilliviruses, it represents morbilliviruses H protein prototype (Hashiguchi et al. 2011). Hashiguchi et al. (Hashiguchi et al. 2007) have generated the MV hemagglutinin crystal structure, reporting that the cubic shaped β-propeller structure of H receptor binding head domain forms a homodimer. They found that protein N-linked sugars seem to affect dimer orientation and shield most of the protein structure, while the MVs highly conserved putative receptor-binding site is located in the protein unshielded region. They suggested that these H N-linked sugar moieties do not favor MVs host immunity evasion, but, instead, critically modulate virus–cell receptor interaction and the antibody response. Based on these structural data, in 2011 (Hashiguchi et al. 2011), they performed the MV-H–SLAM complex crystallization using the 30-140 residues marmoset (ma) SLAM V-set Ig domain and the MV H 149-617 residues head domain. They also engineered the fusion construct to improve diffractions efficiency by connecting a shorter MV-H head domain of 184–607 residues to a N102H and R108Y substituted maSLAM-V via a 12 residues (coding for aa: Gly-Gly-Gly-Ser) flexible linker. The N102H and R108Y substitutions are ment to replace original residues that probably interfered in the original crystal complex packing. They further introduced an additional MV H L482R substitution in order to improve diffraction resolution up to 3.15 Å. Zhang et al. (X. Zhang et al. 2013), instead, elucidated the molecular interaction between MV H and Nectin 4 by solving the crystal structure of Nectin 4 V-set Ig domain (Nectin-4v) complexed to MV H protein. The crystallized complex demonstrated that Nectin 4 N-terminal domain binds MV H β4-β5 groove through extensive hydrophobic interactions. Comparison with the previously studied MV H-SLAM crystal structure highlited specific MV H-Nectin 4 complex binding sites and the MV-H β4-β5 groove hydrophobic pocket shared in both receptors binding, thus representing a potential antiviral drugs target. Based on these MV H structural data, it has been seen that PPRV Nigeria/75/1 strain H protein head portion is sufficiently similar to MV H structure and that most of MV H protein surface side chains involved into both SLAM and Nectin 4 appear to be well conserved in PPRV (*Fig.21*). MV and PPRV side chains aminoacidic sequences, involved in specific SLAM interaction, in fact, only differ for two aminoacidic residues: Pro545, Phe483 in MV and Thr545, Leu483 in PPRV. However, there are extensive aminoacidic differences along the 191–195 stretch

of the two proteins where the H-SLAM interaction is mediated by the polypeptide backbones. This stretch has been proved to be crucial in determining PPRV H selectivity for species specific SLAM receptors, where even a single amino acid change can be enough to greatly increase its affinity for the hSLAM receptor, not commonly used by PPRV (M. D. Baron et al. 2016).

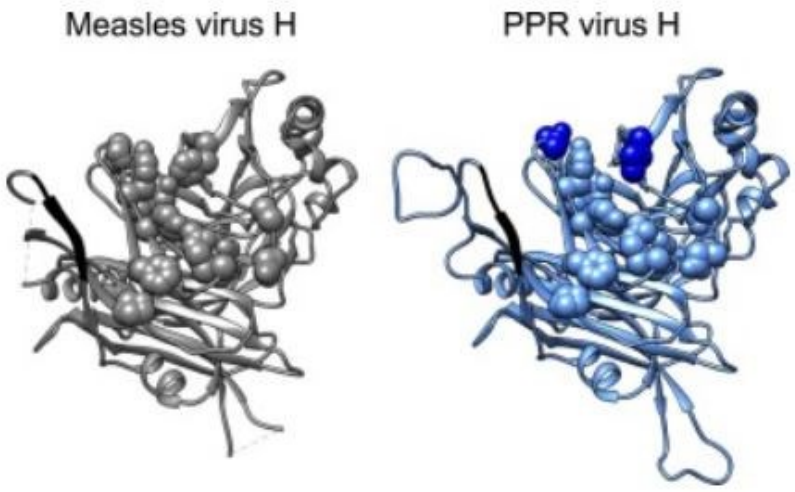

Figure 21. **Comparison of the conserved SLAM-interacting surface residues of Measles virus and Nigeria/75/1 PPR virus H proteins crystal structures.** Adapted from (M. D. Baron et al. 2016) Spherical motifs represent the amino acid stretches involved in SLAM receptor binding. Black motifs represent the protein backbones mediating the H-SLAM interaction. PPRV H protein dark blue spherical motifs represent critical amino acid residues 483 and 545 differing from MV H structure.

However, it remains unclear whether these morbilliviruses preferences for certain species-specific SLAM receptor rather than others might be due host pathogenesis specificity or not. PPRV is known to cause disease in sheep and goats and to give only subclinical infection in cattle and buffalo where anti-PPRV antibodies can be detected in their sera, but it would be of interest to understand if its receptor preferences might be critical in defining these outcome differences. This understanding might help in assessing morbilliviruses mutations risks in using more efficiently certain SLAM receptors and whether they might cause disease in novel hosts. Moreover, since there are evidences of MV H receptor binding region being a conserved neutralizing epitope and since the morbilliviruses H proteins surface containing both SLAM and Nectin 4 binding sites does not easily mutate, these data might explain why

morbilliviruses all show a single serotype and undergo little antigenic drift over many decades. It would be interesting to study whether this epitope dominance might also be valid for PPRV sheep and goats serotypes, as it is seen that a strong antibody eliciting epitope can can be so in one species but not necessarily as strong in another one (M. D. Baron et al. 2016).

Liang et al. (Liang et al. 2016) exploited the available MV H protein three-Dimensional (3D) structure, reported by Colf et al. in 2007 (Colf, Juo, e Garcia 2007), in order to predict a reliable PPRV H structural model, clarify its interaction with SLAM receptor and identify PPRV H specific epitopes eliciting the host neutralizing antibodies response. The MV H-maSLAM 3D structural complex has been used as template for PPRV Hv-sheep (sh) SLAM complex homology modeling. The resulting data showed that PPRV H protein is a six blades (B1-B6) β propeller folded monomer surrounding a big cavity where SLAM interaction occurs. Each blade cosists of four antiparallel β strands (S1-S4) and all six blades are connected through the loops between the S4 of the previous blade and the S1 of the next one. As shown in *Fig.22*, the PPRV H head domain of the predicted PPRV Hv-shSLAM specific structural conformation exhibits the six bladed β-propeller folding, forming a monomer, that allows H protein interaction to the SLAM cellular receptor (purple coloured), which exploits a typical β-sandwich structure, composed of BED and AGFCC' β-sheets.

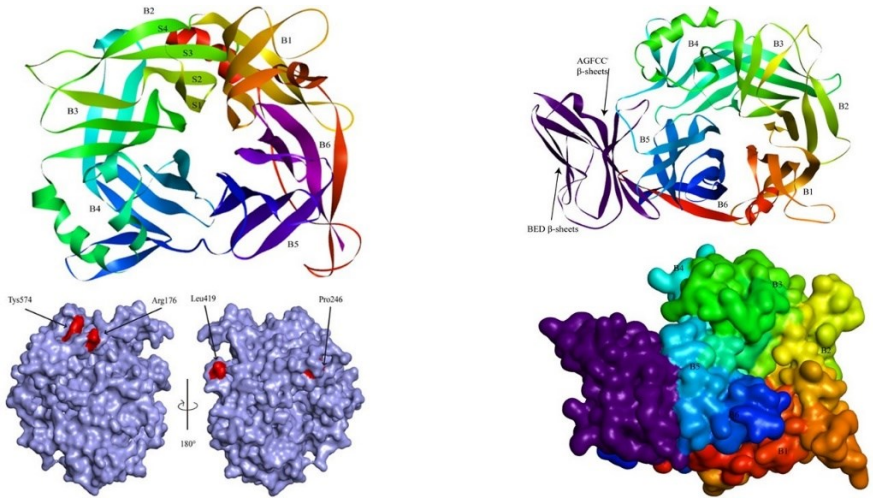

Figure 22. **3D cartoon and solvent drawing representation of the B1-B6 blades β-propeller PPRV Hv predicted folding structure.** Adapted from (Liang et al. 2016)

The B1-B6 blades β-propeller structure sorrounding a large binding cavity (left) and, in purple, the β-sandwich shSLAM structure complexed to PPRV Hv protein (right).

Their results showed that in PPRV Hv-shSLAM complex, the interactions occur in the groove between PPRV Hv head domain B4-B5 blades and shSLAM membrane-distal ectodomain AGFCC' β-sheets. Specifically, shSLAM C β-sheet, C′β-sheet, and the loop in between represent the interface core regions. The interface interaction is aboundantly mediated by hydrophobic and electrostatic interactions, and hydrogen bonds. Four aminoacidic regions of both molecules playing a key role in the structural complex binding interface have been identified (*Fig.23*).

- Region 1, characterized by an intermolecular β-sheet constitued by PPRV Hv Arg191-Arg195 and shSLAM Val126-Ser132 amino acid strands, plus the hydrogen bond between PPRV Hv Arg191 and shSLAM Ser50 amino acids.
- Region 2, determined by salt bridges between PPRV Hv Asp505 and Asp507 and shSLAM Lys78 amino acids and two hydrophobic interactions between PPRV Hv Arg503 and shSLAM Lys77 and Leu92 amino acids.
- Region 3, formed by non-covalent Pi interactions between PPRV Hv Phe552-Pro554 and shSLAM Val128 and His130 amino acids, as well as hydrophobic interactions between PPRV Hv Ser550, Phe552-Pro554 and Arg556 and shSLAM Lys76, His130, Val128, Glu124, and Val126 amino acids respectively.
- Region 4, formed by several non-covalent aromatic stacking between PPRV Hv Tyr541 and Tyr543 and shSLAM Phe119 amino acids, non-covalent Pi interactions between PPRV Hv Arg553 and shSLAM His62 amino acids, and hydrogen bonds between PPRV Hv Asp530 and Arg533 and shSLAM Lys78 and Glu123 amino acids.

The comparison of proteins-ligand interaction energy showed that the interaction mechanism and energy of both viruses and both SLAM receptors were very similar. Moreover, Liang et al. data showed that PPRVHv-shSLAM and MVH-maSLAM binding interfaces were consistent to the previous results. Of all the interfacial interactions, PPRVHv Phe552, Arg533, Arg191, Tyr543, Arg503, Asp505 and Asp507 and shSLAM Lys78, Lys76, His130, His62, Leu64, Val128 and Glu123 were identified to play a crucial role in PPRVHv-shSLAM complex interaction.

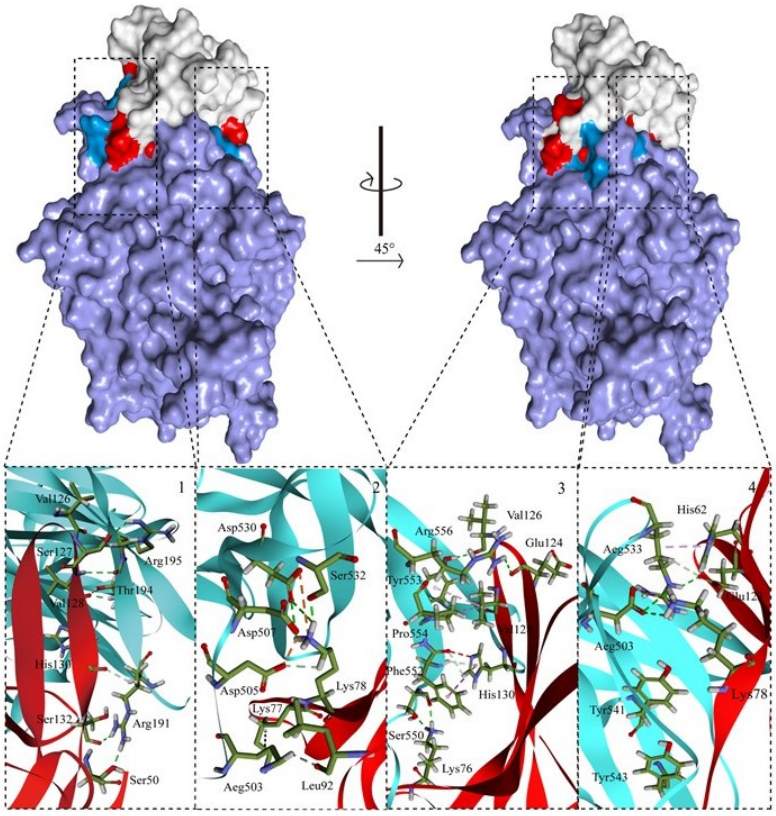

***Figure 23*. Solvent and stick drawing representations of the four small amino acidic regions involved into PPRV Hv (blue coloured)-sh SLAM (red coloured) complex binding interface**. Adapted from (Liang et al. 2016)

Yusuke et al. (Hashiguchi, Maenaka, e Yanagi 2011) exploited both the available MV H–SLAM and MV H-CD46 crystal structures and functional studies to reveal the mechanism behind the long lasting efficiency of the via MV vaccine. Both SLAM and CD46 receptors were proven to target a small exposed region located in MV H epitope compact domain. Subsequent mutagenesis studies demonstrated that also Nectin 4 receptor target this H region.

RNA dependent RNA large polymerase, L. L represents the largest but less expressed PPRV protein and has a predicted molecular weight of 247,30 KDa. Together with P co-factor, it processes genome replication and transcription and mRNAs capping ad polyadenilation. L shares three conserved domains among paramyxoviruses:

- The first domain is represented by residues 1 to 606 and binds RNA.
- The second domain goes from residue 650 to 1694 and represents L protein active site.

- The third domain goes from residue 1717 to 2183 and posseses kinase activity.

Non structural proteins

PPRV C and V non structural proteins result to be pretty conserved among different morbilliviruses and paramyxoviruses, playing several different roles during the viral replication process and defining PPRV virulence, thus faciliting its immune system escape.

Non structural protein C. PPRV C non structural protein has about 20 KDa of molecular weight and it is synthesized by alternative reading frame, using a different start codon of P gene ORF. During viral replication, the first C proteins are transcribed using the second AUG triplette of P ORF while the subsequent C proteins are generated through P mRNA editing at the 3'-AAUUUUUCCCGUGUC-5' conserved site through the addition of G nucleotides, causing a translational frame shift (Schneider, Kaelin, e Billeter 1997; Ward et al. 2011; Kumar et al. 2014a). Compared to P protein, C one does not show phosphorilation and can be found either in the host cell nucleous or cytoplasm. C protein mediates several functions through the binding and interaction with the L protein, such as modulation of RNA dependent RNA large polymerase activity and viral replication. It has also been seen that C protein plays an important role in increasing PPRV virulence by modulating the host immune system response through the blocking of INFs I induction, being very active during the early infection stage (Kumar et al. 2014a). However, the molecular mechanism through which C proteins interfere with INFs induction remains unclear. It should be determined whether the blocking occurs through a direct interference on INFs transcription or through an indirect regulation of defective interfering viral particles early production, or both (M. D. Baron et al. 2016).

Non structural protein V. PPRV V non structural protein is synthesized through P mRNA frame shift, due to the insertion of G residues into the conserved P mRNA editing site. V protein has a predicted molecular weight of 32,28 KDa and compared to C non structural protein, it appears phosphorilated and capable of binding and interacting with both L and N proteins, regulating viral genome replication and transcription. V protein possesses two domains covering dinstinct functions:

- The N terminal domain, little conserved among paramyxoviruses, implied into host cell STAT1 and STAT2 binding.
- The C terminal, cysteine-rich, V specific domain, higly conserved among all paramyxoviruses, implied into the blocking of type I INFs (INFalpha and beta) signaling through interaction with the two host cell INF receptor associated kinases: Janus Kinase 1, JAK1, and Tyrosine Kinase 2, Tyk2.

All V protein interactions are involved in feedback mechanisms that aim to favour virus replication by regulating host cell processes and blocking INFs induction and activities at various points across the pathways. V protein inhibition of JAK1 and Tyk2, infact, also blocks STATs phosphorylation while its binding to STAT1 also blocks type II INF (INFgamma) action. Moreover, morbilliviruses V proteins have been reported to block the MDA5 Pathogen Related Receptor (PRR), thus inhibiting INFs induction. In addition, PPRV V protein has been observed to bind LGP2 and RIG-I MDA5-related proteins, even if these proteins binding functional roles are not clear. LPG2 does not seem to enhance the binding to RIG-I and it is known to play both a positive/negative regulatory role in INFs induction by viruses and to be essential for RIG-I and MDA5-mediated antiviral activities (M. D. Baron et al. 2016).
To better understand C and V non structural proteins role to block INFs induction, two recombinant PPRVs deleted for C and V genes, respectively, have been generated. Interestingly, while the V-knockout PPRV was still able to inhibit INFs induction as the WT virus, the C-knockout PPRV was not. These data suggest that the morbilliviruses V proteins blocking of MDA5-mediated INFs induction is primarily related to the late stages of infection, while C proteins apper to be related to the initial stages (M. D. Baron et al. 2016).

Replication cycle and host immune response

PPRV replicative cycle occurs in different subsequent phases, starting from the virus attachment and its adsorbition to the host cell and the subsequent exploitation of the cellular replicative machinery to transcribe its genome in order to express all the viral components necessary to assembly and generate the new viral progeny (*Fig.24*). *In vitro* PPRV replicative cycle takes place in 6 to 8 hours (Kumar et al. 2013; 2014a).

Attachment. Viral replication cycle first step involves PPRV attack to the host cell surface, mediated by the interaction between PPRV H surface protein to the host cell surface receptor. There are two cell surface receptor molecules involved in PPRV H protein binding: the immune cells marker "signalling lymphocyte activation molecule", SLAM, also called CD150 marker (Seki et al. 2003; Adombi et al. 2011; Parida et al. 2015b) specifically expressed on lymphocytic and dendritic immune cells surface and the epithelial cells receptor Nectin-4 (Birch et al. 2013; Parida et al. 2015b). Since PPRV usually infects hosts through the respiratory tract, where first immune defense cells, such as dendritic cells and macrophages, are present, SLAM receptors represent the mostly exploited molecules for viral attachment, while Nectin-4 receptors are exploited for subsequent viral amplification and dissemination (Noyce et al. 2011; Kumar et al. 2014a). As evidence Pawar et al. have shown that the viral replication amount is directly proportional to SLAM receptor expression on peripheral blood mononuclear cells, PBMCs (Pawar, Dhinakar Raj, e Balachandran 2008), while Adombi et al. proved that PPRV isolation from infected samples is more efficient when using recombinant monkey cells expressing goat SLAM receptors intead of cattle ones (Adombi et al. 2011). However, some ubiquitour host cells receptors, alternative to SLAM and Nectin-4 ones, might be exploited for viral entry as some morbilliviruses, such as Measles virus, MV, have been seen able to infect neuronal, endothelial cells (Sato et al. 2012; Kumar et al. 2014a) and others different cell lines (Hashimoto et al. 2002; Fujita et al. 2007; Kumar et al. 2014a) not expressing neither SLAM or Nectin-4.

Adsorbition. PPRV adsorption and the consequent release of the viral genome into the host cell cytoplasm is mediated by HR1 and HR2 F protein domains fusogenic activity, triggered by viral H protein and cellular receptor interaction, causing fusion between viral and host cells membranes (Parida et al. 2015b). The viral genome is released incapsulated into the ribonucleoprotein complex in order to be protected from the host cell RNAses (Parida et al. 2015b).

Transcription and replication. Viral genome transcription and viral genes expression is started and operated into the host cell cytoplasm by the viral RNA dependent RNA large polymerase, RdRp, already present into the infecting PPRV. Right after viral adsorbition and genome release into the cytoplasm, RdRp starts viral mRNAs transcription by binding the genome promoter, GP. As RdRp can only start genome transcription at GP, it runs along the

genome until it reaches the gene to be expressed and then released its sequence once it comes to the intergenic region, IG. This viral genes transcriptional mechanism allows to keep under control the amount of each protein type produced and to create a precise protein gradient, where N protein, which is the closest to the GP, results to be the most transcribed one, while in contrast, L protein, which is the farest from the GP, is the one produced in the lowest amount. Each viral mRNAs produced is processed by RdRp in order to be 3' poly adenilated and 5' capped and then stably transported into the host cell cytoplasm to the ribosomes where they are translated into proteins. At some point during viral transcription, RdRp interrupts viral mRNAs production and starts to synthetize full lenght positive sense RNA antigenomes; this functional switch might be directly related with unassembled N protein accumulation into the host cell cytoplasm (Wertz, Perepelitsa, e Ball 1998). However a second theory hypotetizes the existence of two different RdRp types, that separately control and direct genes transcription and genome synthesis (Gupta, Shaji, e Banerjee 2003; Kolakofsky et al. 2004). Once the positive sense antigenomes are synthetized, RdRp progresses PPRV genome replication by binding the 3' antigenomic promoter, AGP, and producing new full lenght negative sense RNA genomes, ready to be incapsulated into ribonucleoprotein complexes (Kumar et al. 2014a; Parida et al. 2015b).

Assembly and release. The viral progeny assembly takes places at determined host cell membrane sites where viral budding will take place, following viral proteins and RNP complexes synthesis (Harrison, Sakaguchi, e Schmitt 2010; Kumar et al. 2014a). Assembly and release of all paramyxoviruses is guided by the matrix protein, M, which is infact the most produced one (Kondo et al. 1993; Kumar et al. 2014a; Parida et al. 2015b), by binding together viral structural elements, such as H and F glycoproteins and RNP complexes, with the host cell membrane, on the cytoplasmatic side of the plasma membrane. The other viral proteins, glycoproteins H and F and C protein, cooperate with M protein during viral assembly process, while cellular endosomial sorting complexes required for transport, ESCRT determines virions budding and release (Vincent, Gerlier, e Manié 2000; Shaikh e Crowe 2013; Kumar et al. 2014a).

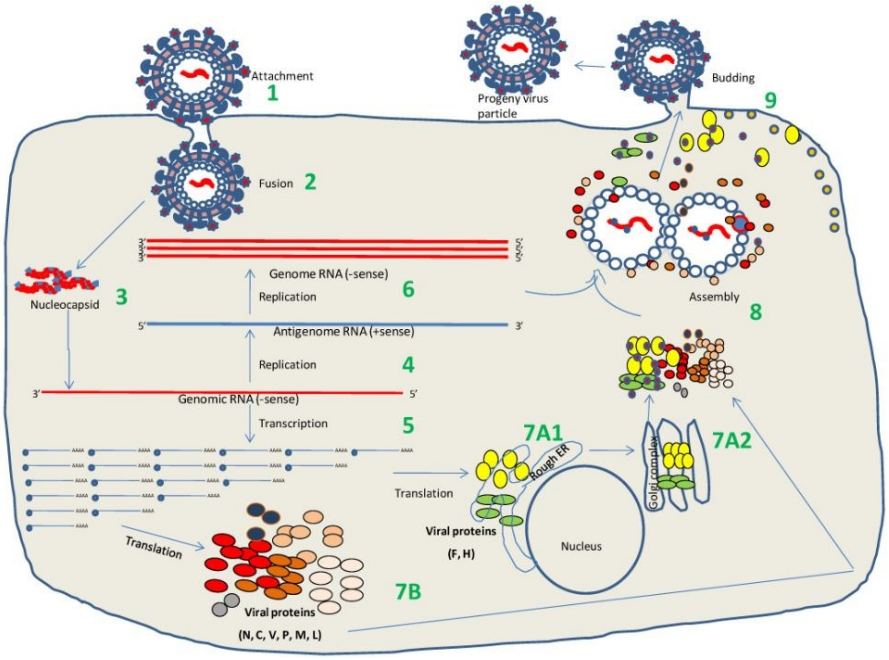

***Figure 24*. Schematic representation of PPRV replication cycle phases**. Adapted from (Kumar et al. 2014a)

PPRV replication cycle phases: attachment (1), adsorbiton (2, 3), viral genome replication (4, 6) and viral genes transcription and translation (5, 7A1, 7A2,7B), assembly and release (8, 9).

Host immune response

During a viral infection the host organism immune system first interacts with the pathogen structures and responds through the establishment of an innate viral resistant state, which, in turn, stimulates an adaptive immune response. PPRV mainly infects host immune system cells and establishes a lymphotropic infection, causing host immunosuppression and leucopenia (Rajak et al. 2005; Pope et al. 2013; Parida et al. 2015b).

Innate immunity. To date, the effector network through which PPRV triggers and stimulate the innate immune system remains very poorly understood. However, following PPRV infection, goats have been observed to respond with a classic inflammatory response, characterize by high cytokine levels expression. Interferons (INFs) represent the main cytokine group involved into the establishment of a viral resistant state. Although morbilliviruses are well known to inhibit INFs signaling, recent studies on several morbilliviruses clarified that V protein blocks type I INFs activities but the interference with type II INF greatly varies among different morbilliviruses (Kumar et al. 2014b). Different cell types, including lymphocytes, produce type I INFs (INFα and INFβ) which represent a

first barrier against viral infection as they interfere with viral replication by alarming the nearby uninfected cells to protect themself against the pathogens and by inducing virus digestion into infected cells through JAKs (JAK1 and JAK2) and STATs (STAT1 and STAT2) pathways. Type II INF (INFγ) is, instead, produced by T and NK cells and mediates its antiviral response through JAKs and STAT1 pathways (Randall e Goodbourn 2008; Platanias 2005; Kumar et al. 2014a). PPRV infection triggers several different cytokines, primarly type I and II interferons and IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12 interleukins (Kumar et al. 2014a; Jana Baron et al. 2014; Atmaca e Kul 2012).

Adaptive immunity. Morbilliviruses infection usually induce a massive anti-viral immune response, comprehensive of cell-mediated and humural responses, mainly directed against H, F and N proteins. H and F envelope glycoproteins are known to strongly induce protective nautralizing antibodies, while N viral protein, which represent the most abundant one, does not. However it has been found that N protein strongly stimulate cell-mediated immunity, which contributes to protect from reinfection. Althought antibodies are considered the most likely effectors for inducing immune protection, studies on rinderpest showed that antibodies alone are not able to infer protection, suggesting that cell-mediated response in association to humoral immunity is crucial to provide an effective immune protection. Attenuates morbillivirus vaccines demonstrated to induce both humoral and cell-mediated immunities. However, it remains unclear which immune effectors, among systemic neutralizing antibodies and cytotoxic T cells, are responsible for PPRV vaccine protection.

Diagnostics

Since PPRV produces clinical signs similar to other diseases, thus being easily confused, PPR control and eradication program needs validating specific diagnostic tools and reagents. Virus isolation, antigens detection, PCRs and specific antibodies indirect detection represent already developed diagnostic techniques.

Virus detection depends upon samples of infected animals showing the disease clinical signs, as well as from those who rarely recover. Despite new diagnostic methods showed that virus shedding may occur 1 to 3 days before the clinical signs appearance, the virological investigations time window, through RT-PCR or ELISA techniques, is very short.

Samples with greatest virological diagnostic value are mostly collected during the initial mucous membranes flaking phase. However, serological analysis can be done at any time

for at least three years after seroconversion occurring. PPRV mainly locates in lymphoid tissues and respiratory, digestive, and lymphoid systems epithelium, thus being easily isolated from blood stream and lungs-, lymph nodes-, small intestine-, and spleen-biopsies during disease initial stages, but also from post-mortem samples.
Nonetheless, as ocular and nasal discharge samples contain high viral loads and can be more safely collected compared to blood samples, they represent the common site for viral RNA detection. Swabs collection is simple and does not cause pain to animals, increasing samples collection breeders compliance. However, wild animals diagnosis remains difficult, since their capture and containment are laborious and expensive. PPRV diagnostic for non-invasive samples collection, like feces, are thus now being provided, in order to include wildlife into PPR surveillance (M. D. Baron et al. 2016).

Virus isolation. Initial PPRV isolations have been conducted on primary lamb kidney and ovine skin tissues cell cultures. Later on, Hamdy et al. (Hamdy et al. 1976), have introduced Vero cells, that represent the most commonly employied cell line for PPRV isolation. PPRV isolation sensitivity has then been further increased by making the virus growing in lamb or goat kidney cells, and subsequently it has also been adapted to grow in Madin-Darby Bovine Kidney (MDBK) epithelial, Baby Hamster Kidney-21 (BHK-21) fibroblast and other continuous cell lines. Nonetheless, Vero cells deriving from the African green monkey kidney, remained the most widely used cell line for PPRV isolation, untill goat or dog Signaling Lymphocytic Activation Molecule (SLAM) discovery. SLAM, also known as CD150, is a lymphocite surface protein receptor, present in morbillivirus susceptible hosts, that is preferentially exploited by WT MV, CDV and RPV morbilliviruses for host cell binding. Despite lymphoid tissues represent morbilliviruses major replication site, they can infect and replicate also in other organ epithelial cells and lung and kidney cells, by binding different cell receptors types. However, viral replication in these non-lymphoid cells is 100 to 1000 times lower compared to lymphoid cells, but since they are easily *in vitro* cultured, they have been preferred for morbillivirus isolation. In particular, initially PPRV has been widely isolated and mantained on bovine, sheep and goat kidney primary cells, but since primary cell lines present considerable drawbacks, the African green monkey kidney (Vero) cell line has then been preferred. However, as well as for other morbilliviruses, Vero cells allow very low PPRV isolation, requiring multiple, subsequent blind culture passages, taking several weeks before the CPE appearance. Following SLAM discovery as potential protein for PPRV isolation and propagation, a CV1 cell line, deriving from the African green

monkey kidney, and expressing the goat SLAM receptor, has been developed (Mantip, Shamaki, e Farougou 2019). Experiments on monkey CV1 cell line expressing goat SLAM, highlighted its efficiency in PPRV isolation from pathological samples, suggesting that SLAM, as well as for MV, CDV and RPV, is also exploited by PPRV (Adombi et al. 2011, 1). The recent use of modified cell lines expressing SLAM has highly shortened PPRV isolation from pathological samples and its propagation, comparing to the previous methods. Another explored technique for PPRV isolation is based on Vero cells expressing nectin-4, but since it requires high costs, specialized facilities and expanded times, it is not appropriate for disease outbreacks alerting, either in endemic than not contaminated areas (M. D. Baron et al. 2016).

Serological diagnosis

Antigens detection. Sensitive Enzyme-Linked Immunosorbent Assay (ELISA) for PPRV N protein detection, referred to as sandwich ELISA (sELISA) and Immunocapture ELISA (ICE), have been developed. The developed sELISA assay is based on a specific anti-N-mAb directed against a PPRV-N epitope, routinely used for PPRV clinical prevalence surveillance or PPRV antigens detection in infected goats tissues and secretions. The more widely used ICE, allows rapid differential diagnosis of PPRV and RPV, which is of great importance since they both have similar geographical distributions and may affect the same animal species. ICE, similarly to sELISA, is based on a pan-morbillivirus anti-N-mAb against an N PPRV/RPV cross-reactive epitope, and it takes 2 hours to give a reliable result on samples that have been manteined for a week at RT, with a maximum 50% lower response. This assay shows high specificity and sensitivity even for low titer virus content samples, with RT-PCR equivalent performances, and it is exploitable for PPRV and RPV routine diagnosis from a wide range of field samples, such as ocular, nasal and rectal swabs, tissue samples and PBMCs. ICE is also exploited for outbreacks confirmation in infected sheep and goats, but also from infected camels too. Compared to sELISA, ICE present a 4% more of diagnostic sensitivity and specificity (M. D. Baron et al. 2016; Mantip, Shamaki, e Farougou 2019).

Antibody detection. Antibody detection tests allow endemic and infected countries to certainly evaluate a disease prevalence. Since they well adapt to large-scale researches, ELISA tests are the method of choice for antibody detection. PPRV H- or N Ab-based

competiton ELISA (C-ELISA) tests present high correlation to the Virus Neutralization Test (VNT), which is limited by high costs and a timing of one-two weeks.
PPRV H mAb assays are based on virus crude antigens (derived from either PPRV Sungri/96 or Nigeria75/1 vaccinal strains) coated plates. In order to assure tests safety and avoid any risk of residual live virus contamination, standardized ELISAs based on recombinant PPRV antigens have been developed. These assays are safe and exploytable for routine PPRV detection in both endemic and virus-free areas. Since it is not able to recreate PPRV H protein normal folding, as in mammalian cells, baculovirus system is not exploitable for its expression. Baron et al. (Jana Baron e Baron 2015) have, therefore, created a PPRV helper cell dependent by removing the entire RNA polymerase (L) gene from viral genome and complementing it with the same protein constitutively expressed in a cell line. The L-deleted PPRV can thus efficiently grows in the L-expressing cell line but not in others. This is a biosafe system that allows the production of sufficient L-deleted virus quantities, usable as antigen in ELISA test, without the need of high level biocontainment facilities.
As it represents PPRV most produced protein, the immunogenic N protein constitues one of the best antigen candidate for standardized diagnostic tests. Even if anti-N Abs are not neutralizing, many N-based ELISAs have widely been developed, assuming that under *in vivo* vaccination or natural infections, the host immune response is stronger and protective. Most of the developed full-length or truncated PPRV N proteins exploited in diagnostic assays, are expressed into baculoviruses or *E. coli* heterologous systems, with no folding problems (M. D. Baron et al. 2016).

PCRs. Virus isolation and serological techniques represent morbilliviruses conventional laboratory diagnosis to detect viral presence in submitted samples, but they are limited by the fact that they are not employable for detection on decomposed field samples. In these cases, the repetitive cycles of DNA denaturation, primer annealing and target DNA sequence extension, performed during the Polymerase Chain Reaction (PCR) method, allow the *in vitro* specific detection of viral target sequences from poorly conserved field samples. To date, PCR represents the most widely exploited moulecular laboratory technique for disease diagnosis. Since morbilliviruses genomes consist of a ssRNA molecule, the two-step Reverse Transcription PCR (RT-PCR) reaction is needed in order to first retro-transcribe the genomic ribonucleic acids into complementary DNAs (cDNAs). RT-PCRs can rapidly and efficiently detect morbillivirus-specific RNAs from animal samples, and they have been proved to be especially useful for detection in marine mammal samples (Mantip, Shamaki,

e Farougou 2019). Following RPV eradication, the interest and feasability for PPR diagnosis and control have been increased by modern technologies advancement and molecular biology tools avaibility. Particularly, considerable efforts have been spent on nucleic acid tests development for PPRV rapid detection and identification. RT-PCRs based on morbillivirus P universal and F specific primer sets have been performed from some researches to discriminate between PPRV and RPV. In order to distinguish among all morbilliviruses, both genus-specific and universal morbilliviruses primer sets have been generated (Mantip, Shamaki, e Farougou 2019). The amplicon size, sequence and specificity must be selectively confirmed to exclude cross-reactivity phenomena with symptomatically or phylogenetically related morbilliviruses, thus primers type choice, represents a key point in nucleic acids diagnostic detection. Since SYBR Green, the most exploited dye for DNA quantification in conventional RT-PCRs, is capable of binding any type of dsDNAs present in the PCR reaction, primers need to be carefully designed. Conventional RT-PCRs targeting PPRV F and N genes have been extensively applied and validated for virus multiple lineages, but recently, higher sensitive one- and/or two-step RT-PCR assays, such as TaqMan-based RT-PCRs, for PPRV detection and quantification have been reported, providing several advantages, being more rapid and suitable to standardization, and minimizing cross-contamination.

Since N gene is located at genome 3' end, it represents PPRV most expressed gene, thus N-based RT-PCR detection systems are the most exploited assays for PPRV detection on clinical samples. Despite it is not employed in diagnostics, Keita et al. (Keita et al. 2008) have developed a SYBR Green based assay for morbilliviruses N gene specific targeting. TaqMan assays, based on hybridization probes and fluorescence resonance energy transfer for nucleic acids quantification, are commonly employed as alterantive techniques to SYBR Green. The first one-step PPRV N gene-based TaqMan RT-PCR assay has been developed in 2008 by Bao et al. (Bao et al. 2008). It has been the first TaqMan-based assay used for PPRV four lineages evaluation and confirmation on clinical samples, coming from distinct endemic areas. Primers and probe are designed based on lineage IV PPRV-Turkey 2000 strain N gene sequence and test specificity has been assessed by analyzing different morbillivirus RNAs, including RPV, with none of these viruses testing positive. This assay detection limit was found to be 8.1 RNA copies per reaction. Thirty field tissue samples have been screened for PPRV detection by using both Bao TaqMan RT-PCR and conventional RT-PCRs, turning out that TaqMan-based assay increase the detection rate from 46.7% to 73.3% and the detection sensitivity of one log unit. Afterwards, in 2010, Kwiatek et al.

(Kwiatek et al. 2010), developed a second one-step PPRV N gene-based TaqMan RT-PCR for all four viral lineages RNA detection. Primers and probe are specifically designed based on PPRV/Nigeria 75/1strain N gene terminal sequence, corresponding to the C-terminal hypervariable 399-523/525 protein amino acids. PPRV phylogenetically or symptomatically related morbilliviruses, such as rinderpest, bluetongue, and bovine viral diarrhea viruses, are thus selectively not detected. However, lower specificity occurs with PPRV lineage III, based on nucleotide mismatches within the probe region. Compared to conventional RT-PCRs, Kwiatek assay is able to detect 32 minimum RNA copies per reaction, with a 39 Ct corrisponding value. More precisely a positive 33.89 Ct value can still be obtained with only 12 RNA copies/ μL. This assay can detect 20% more positive field specimens with viral RNA content, compared to conventional PCR methods. Moreover, depending on the PPRV lineage involved, the test detection sensitivity is also increased to three log copies. Comparison with Bao et al. RT-PCR assay, in term of PPRV lineage detectability based on a proficiency panel, has also been made. Both assays performs are comparable, since they are both able to detect all PPRV four lineages, even if Kwiatek assay shows a slight improve on lineage II detection. However, this last test proved significantly faster performance, thus being better adapted for numerous specimens monitoring. Similar RT-PCR assays, targeting PPRV N gene terminal conserved regions, have been developed by Batten et al. (Batten et al. 2011) and Polci et al. (Polci et al. 2015). The first group developed a rapid and high throughput RT-PCR assay for specific PPRV detection, using robotic RNA extraction. The assay demonstrated to detect all four PPRV genetic lineages from field nasal and ocular swabs, tissues and blood samples, with a lowest detection limit of about ten RNA copies per reaction. The second group developed a higher affinity but more expensive newly designed duplex RT-PCR, based on primer set and TaqMan probe designed on PPRV N conserved region. The commercial heterologous Armored RNA(®) has been used as internal positive control, allowing the identification of five false-negative results, occurred by amplification failure. This assay has been evaluated on all PPRV lineages, except for lineage III, showing a detection limit of twenty RNA copies/μL with 95% of probability. Overall, the four PPRV N gene-based RT-PCR assays above, proved high specificity and comparable analytical sensibility with very low detection limits. Finally, more recently, a one step, SYBR Green-based RT-PCR for PPRV detection, has been developed by Balamurugan et al. (Balamurugan, Sen, Venkatesan, Yadav, et al. 2012). Their assay is based on the use of PPRV M-gene primers with a Hexachloro-fluorescein (HEX) labeled fluorescent probe, that amplifies a 124bp gene fragment at the melting temperature of 78˚C. Their results show

higher PPRV diagnostic values compared to conventional PCR and ELISA sandwich, enabling virus shedding detection in preclinical swabs 3 to 20 days post-infection, with a vaccines and field strains 0.0001 $TCID_{50}$/mL detection limit. The established SYBR green RT-PCR is a valid alternative to TaqMan RT-PCRs, emploiable for rapid clinical diagnosis with low contamination risk (M. D. Baron et al. 2016).

All these mentioned above diagnostic tests allow both the disease spread evaluation into disease-free areas, and the official declare of which are the contagious-free countries. In the OIE "Manual of diagnostic tests and vaccines for terrestrial animals 2019" («Access Online: OIE - World Organisation for Animal Health» s.d.), chapter 2.7.11, these available diagnostic tests have been mainly classified into two groups of recommended (+++) and suitable (++) methods (*Fig.25*). While virus isolation is a suitable but not recommended method for clinical cases confirmation as its application requires a long time, any tests based on either PPRV proteins or genes are higly recommended. Both virus neutralization tests (VNTs) and ELISAs, are serological methods detecting anti-PPRV Abs that can define whether populations or individuals previously infected are disease-free or currently still infected and define the immune status following vaccination. Specifically, VNTs are always recommended (+++) for clinical diagnosis, especially when there is the need of defining whether areas are disease-free, while ELISAs are only recommended for disease surveillance and seromonitoring (M. D. Baron et al. 2016).

		Purpose				
Method	Target	Confirmation of Clinical Cases	Population Freedom from Infection	Individual Freedom from Infection	Prevalence of Infection—Surveillance	Immune Status in Individual Animals—Vaccination
ICE-ELISA	Protein	+++				
RT-PCR	Gene	+++				
QRT-PCR	Gene	+++				
Virus isolation	Virus	++				
VNT	Antibody		+++	+++	+++	+++
C-ELISA	Antibody		++	++	+++	+++

Figure 25. **Principle available PPRV detection tests and their purposes**. Adapted from (M. D. Baron et al. 2016)

PPRV outbreaks Field Tests

One of the major limitations occurring in the PPR-free areas, where the contagion risk is significant, is the possibility of real time monitoring the disease outbreaks onset, in order to be able to promptly intervene with the appropriate containment measures implemention, bypassing the timing required for the laboratory test results. In addition to be time consuming, laboratory diagnostic tests require skilled personnel, while an efficient field test must be robust, simple and ready to be on real time implemented. Recently, several diagnostic field tests fulfilling these requirements have been developed. In 1999, Bruning et al. (Brüning et al. 1999), developed the first morbilliviruses field test, consisting of a rapid pen-side test for RPV antigen detection from cattle lachrymal fluids, by using the Clearview chromatographic strip test technology. This test can detect viral antigens from lachrymal fluids of both expreimentally and naturally infected cattle, with no cross reactivity with other related morbilliviruses. The test field performance has been carried out in Pakistan, at the Landhi cattle colony, sampling ninety-seven animals, some of them showing various clinical signs, that have been first tested with Clearview and subsequently by immunocapture ELISA (IC ELISA). Nineteen animals resulted positive with Clearview and/or IC ELISA to RPV, among which seventeen were Clearview-positive and fifteen IC ELISA-positive. RT-PCRs further confirmed the nineteen samples positiveness to RPV. Later, Clearview, has been improved for RPV and PPRV detection from live animals eye swabs. Baron et al. (J. Baron et al. 2014), have developed and commercialised an immunochromatographic test for PPRV field diagnosis. Their assay has been tested on ocular and nasal swab samples in both laboratory and field (Ivory Coast, Pakistan, Ethiopia and Uganda) conditions, proving an 84% sensibility compared to PCRs and 95% of specificity. All four PPRV genetic lineages can be specifically detected from the animal swabs at four early days post-infection, when clinical signs are minimal. This test has been easily and positively used for PPR diagnosis in field trials. The results of these field tests are clear and reliable enough to be readily performed on fresh samples in the field infection site, avoiding the risk of samples depletion, which can occurr when specimens have to be sent away for laboratory diagnosis. A recently explored alternative method for field diagnosis of PPRV RNA is the Reverse Transcription Loop-mediated isothermal Amplification (RT-LAMP) assay. Li et al. (Li et al. 2010), developed a one-step, single-tube RT-LAMP assay targeting PPRV M gene sequence, where the amplicons can be naked eye observed. The specificity and sensitivity of their assay have been validated on eight different PPRV strains from different geographical areas, without showing cross-reactivity with other related morbilliviruses. RT-LAMP assay has shown to

be able to detect PPRV RNA on a wide spectrum of positive clinical samples, processing the detection results within one hour. The detected PPRV genetic lineage specificity is consistent with those detected by RT-PCRs, showing a ten fold higher sensitivity.
Based on these data, chromatographic strips and RT-LAMP assays present interesting and practical advantages for their application in field detection, such as their analytical and diagnostic accuracy of real time monitoring with no need for expensive reagents and equipment. However, to date, the RT-LAMP assay emploiment on field is still prevented by the limitations of requiring prior RNA purification from clinical samples with the need of significative extra time and equipment, and the susceptibility to cross-contamination expressing false positive results (M. D. Baron et al. 2016).

PPRV Vaccines

PPRV and other morbilliviruses pathogenesis main characteristic is the establishment of a transient but strong, virus-mediated immunosuppression, increasing host susceptibility to further opportunistic infections that worsen the infection outcome. Immunosuppression is due to viral replication in lymphoid cells and morbilliviruses have developed different strategies to avoid both innate and induced host immune responses. However, the immunosuppressive effect is only transient and the disease recovery usually ends up with the establishment of a specific, long-lasting and effective immunity. Since virus transmission is mainly due by close contact between infected and healthy animals, the first sanitary preventive measures that should be taken are:

- -To prevent the movement and trade of infected livestock towards PPRV infection-free territories.
- -Following the outbreak extintion, to disinfect territories and farmers that have been affected and support farmers who have lost their livestock.

However, considering that PPR pandemic mostly affect developing countries, these measures are hardly implementable. Vaccination, thus represent the primary efficacious strategy to prevent and control PPR in developing pandemic countries (M. D. Baron et al. 2016). In order to achieve PPR control and eradication, it is also important to keep in mind this Tom Barrett et al. phrase: *"If rinderpest becomes a disease of the past, PPR is certainly*

a disease of the future". They hypothetised an adeguate PPR-rinderpest balance in order to plan an efficacious PPR eradication, focusing on several points, such as morbilliviruses relative relationships, comparative molecular biology, infectious diseases pathophysiology, global epidemiologic patterns, countries contributions to the rinderpest control and eradication campaigns, viral immune suppression, and the developed molecular diagnostic approaches. Since the GREP has successfully contained rinderpest infections, it is strongly believed that PPR spread can be similarly controlled and prevented, through vaccination (Munir 2010b).

Inactivated vaccines. Inactivated vaccines have represented the first attempts of vaccination against rinderpest. A formalin inactivated RPV vaccine has been tested, showing just a short-term immunity, with no effective protection for cattle against neither RPV or the other morbilliviruses. The inactivated RPV vaccine was not, thus, suitable for a RPV eradication campaign. An inactivated MV vaccine has also been attempted, with even worse results since its administration caused an increased disease pathology in vaccinated animals when subsequently exposed to WT virus challenge, and just providing short-term protection. Therefore, the best vaccine solutions might be to exploit live attenuated vaccines for endemic regions, like African and Asiatic countries, and inactivated vaccines for non-endemic but disease threatened regions, such as European territories, where veterinary authorities usually not recommend the use of live-attenuated vaccines.

Recently, new PPR inactivated vaccine formulations have been developed and tested. Cosseddu et al. (Cosseddu et al. 2016) developed a PPR inactivated vaccine able to induce a sterile immunity in vaccinated animals, even if after two doses administration, and that was resistant to the virulent PPRV challenge. Ronchi et al. (Ronchi et al. 2016) generated a PPR inactivated vaccine (iPPRVac) by using either a water-in-oil emulsion and a delta inulin adjuvant, alone or combined to the TLR9 agonist oligonucleotide, in its formulations. They tested the immunogenicity formulations in rats models, obtaining that the iPPRVac fomulation with the TLR9- combined delta inulin adjuvant was able to induce 100% seroconversion, after 2 administrations. This formulation was then tested on goats, observing 100% seroconversion on the 9th day after injection, and seroconversion maintenance untill the last experimental 133rd day. These experimental data suggest rat models as useful for vaccine response prediction in goats and show that inactivated vaccines formulated with TLR9- combined delta inulin adjuvant, are a promising alternative vaccine strategy to live

attenuated vaccines for PPR eradication and control in non-endemic countries. However, further *in vivo* studies are required to warrant its use on fields (M. D. Baron et al. 2016).

Live attenuated vaccines. Live attenuated morbilliviruses vaccines generally stimulate a lower immunosuppression effect compared to the respective wild type viruses, but are still capable of inducing a strong effective immune protection. These vaccines key feature has been exploited by Plowright and Ferris (Plowright e Ferris 1962) for the successful Global Rinderpest Eradication Program (GREP). Following Sabin pioneer work (Sabin 1957), they have attenuated RPV through 70-90 culture passages on calf kidney cells, obtaining an effective Tissue Colture Rinderpest Vaccine (TCRV), capable of inducing a high humoral immunity in injected cattle in Kenya, without any clinical reactions. Moreover, the culture-attenuated RPV did not spread from vaccinated animals to healthy susceptible ones, thus being safely used on field (M. D. Baron et al. 2016) Since no homologous PPR vaccines were initially available and considering RPV and PPRV close antigenic relationship, OIE recommended the TCRV use for PPR prophylaxis. Despite no PPRV-neutralizing antibodies were detected, TCRV vaccinated small ruminants succesfully resisted the PPRV challege, being well protected against PPRV infection for about a year. However, since it could have potentially interferred with the GREP, TCRV has then been banned in rinderpest free declared countries, thus a homologous PPR vaccine was necessary. Many attempts have been made in live-attenuated PPR vaccines development, but with no efficient results. In 1962, Gilbert and Monnier (Gilbert e Monnier 1962) have been the first to successfully *in vitro* carried out a PPRV adaptation, by propagating the virus in sheep primary liver cells up to 55 passages. However, despite the virus showed lower pathogenicity, even up to the 65th passage, it was not completely avirulent. These previous PPRV unsuccessful attenuations could be attribuited to the use of an insufficient virus inoculums quantity, or the employed cell culture system types or the used PPRV strains inherent nature. Continuing researches on homologous PPR live-attenuated vaccine development, have finally been successful in 1989, with Diallo et al. (A. Diallo et al. 1989; AC Diallo et al. 2007). They have obtained the first attenuated PPRV through several passages of PPRV/Nigeria/75/1 strain on Vero cell monolayers. At the 20th passage, the virus was just able to induce slight fever in vaccinated animals and it became completely avirulent by the 55 th passage. Moreover, it has also been observed that up to the 63rd passage, when injected into immunized goats, the virus was able to induce a protective antibody response by the 7 th day post-vaccination.

Since its identification, between 1989 and 1996, almost 100,000 sheep and goats have been immunized with PPRV/Nigeria/75/1-based vaccine, with no side effects. Vaccinated

animals showed resistance to challenges with different virulent WT PPRV and did not transmit the challege viruses to susceptible animals in contact with them. PPRV/Nigeria/75/1 vaccinated animals produced three years lasting antibodies, being protected against any virulent PPRV strains (M. D. Baron et al. 2016; Kumar et al. 2017). Initially, PPRV/Nigeria 75/1 has been worldwide efficaciously employed for immunization against all four PPRV lineages, and subsequently, several other PPRV strains have been successfully culture-attenuated. The PPRV/Sungri/96 derives from a goat isolate in Sungri, Himachal Pradesh, India, and has been used to develop a lineage IV-specific vaccine, by attenuating it through ten first colture-passages in B95a (Marmoset lymphoblastoid) cells and subsequently in Vero cells. The virus has became avirulent up to the 56^{th} passage and has exstensively been tested in field small ruminants, proving to be safe and to provide a four years lasting protection, thus being used for PPR mass immunization program in India since 2010. Like PPRV/Sungri/96, at the Tamil Nadu Veterinary and Animal Science University (TANUVAS), in India, other two PPRV strains, the PPRV/Arasur/87 and the PPRV/Coimbatore/97, respectively derived from sheep and goat, have been colture passaged in Vero cells up to the 75 passages to make them avirulent. Both PPRV/Arasur/87 and PPRV/Coimbatore/97 based vaccines showed to be safe and efficacious as Sungri/96 vaccine, thus are being used for PPR immunization in Southern Indian States (Arnab Sen et al. 2010; Kumar et al. 2017). Currently, the PPRV/Nigeria 75/1 (lineage II) and the PPRV/Sungri/96 (lineage IV), represent the most used PPRV live attenuated vaccines and have widely been tested and validated on fields, in addition to viral genomes complete sequencing. PPRV live attenuated vaccines limitation is mainly due to PPR heat sensibility, thus requiring a strict cold chain from generation to delivery. Since most of the territories where PPRV is endemic present hot and humid climates and lack of adapt infrastructures, improved vaccines manteinance strategies are needed (Kumar et al. 2017).

Thermo stable PPRV vaccines. Live attenuated PPRV vaccines general shelf life is of about 1 year at 4˚C, but since PPRV vaccines are mainly intended for African and Southern Asia hot and humid areas, cold chain manteinance is foundamental for their transportation. To this purpose, several improved freeze-drying procedures, enhancing PPR vaccines thermostability, have been developed. For example, in 1990, Mariner et al. (Mariner et al. 1990) have developed a lyophilized Vero-coltured rinderpest vaccine, using sucrose and lactalbumin hydrolysate as stabilizers, with reduced moisture content and increased thermostability. Similarly, in 2000, Worrall et al. (Worrall et al. 2000) developed the ultra

rapid Xerovac method of dehydration and preservation of both live attenuated rinderpest and PPR vaccines. This method reckon on 1:1 virus diluition with a trehalose dehydrate 5% w/V sterile aqueous solution and its subsequent dehydration. The resulting Xerovac-made vaccine is able to resist for 14 days at 45˚C with no significative potency loss. Silva et al. (Silva, Carrondo, e Alves 2011; Silva et al. 2014) have explored several stabilizing strategies to increase PPR vaccines thermostability. They experimented the use of Tris/trehalose stabilizer on both the liquid vaccine formulation, finding an increase of virus half-life up to 21 hours at 37˚C and 1 month at 4˚C, and the lyophilized one, with relative long viral titres maintenance, up to 0.6log loss in 21 months at 4˚C, 0.6log loss in 144h at 37˚C and 1log loss in 120h at 45˚C. They also obtained enhanced vaccine intrinsic stability and 1log higher virus production, by replacing fructose with glucose. Moreover they found out that increased NaCl concentration facilitated virus release from infected cells and lowered the cell-associated virus fraction. In addition, since heavy water has been proved to increase both polio and yellow fever vaccines thermostability, different heavy water-stabilizers combinations have been explored. However, the so explored different heavy water/stabilizers combined vaccines, had just a minimal impact on vaccines thermostability, thus not being effective enough to be employied for the eradication program. PPR control program success will largely depend on an effective vaccine delivery in hot and humid areas, thus the development of a thermostable PPR vaccine is still highly needed. In addition to improved freeze-drying methods and stabilizers, another explored way for vaccine thermostabilization is the use of thermo-adapted (Ta)-PPRVs. For example, the PPRV/India/2003/Jhansi strain has been thermo-adapted by first 25 passages in Vero cells at 37C and subsequent 25 passages in Ta-Vero cells at 40C. The so obtained Ta-PPRV has been tested for sheep and goats vaccination, proving to be an efficacious and safe candidate PPR vaccine. However, further clinical trials need to be done before employing such a vaccine on field.

Like TCRV, PPR live attenuated vaccines are able to provide an effective long lasting immunity in vaccinated animals, after a single immunization. However, in addiction to the fact that inconvenient high costs are required to cover their thermostability, since the induced vaccination and natural infection immune responses are identical, these vaccines don't allow the serological distinguishing between vaccinated and naturally infected and recovered animals. This fact makes the disease epidemiological serosurveillance impossible, thus these vaccines type is not exployitable for control and eradication programs (Kumar et al. 2017).

New generation vaccines

Recombinant DNA technology has allowed the Differentiation of Infected and Vaccinated Animals (DIVA) recombinant vaccines generation, combining together vaccination and serosurveillance activities, and overcoming live attenuated vaccines thermostability limitation. Recombinant vaccines, thus enable epidemic countries to contemporarly implement both disease surveillance and vaccination programs. Marker DIVA vaccines against rinderpest have also been developed but since GREP was almost covered, they have never been implemented in the field. A potential effective DIVA vaccine strategy would be based on the use of recombinant viral vectors expressing PPRV antigens, such as H and/or F surface glycoproteins. These constructs would induce a strong specific immunostimulation only against the expressed recombinant PPRV antigen/s in the vaccinated animals, allowing their distinction from PPRV naturally infected ones, which on the contrary produce antibodies against all the viral antigens. For example, a vaccine based on a recombinant viral construct expressing the H antigen and not the N one, allows the selective identification of vaccinated animals based on H specific antibodies presence and N ones absence. However, to date, since there are several controversial concerning over the genetically manipulated organisms, these vaccines have not yet been licensed beyond their experimental stages. Despite their great potential application for disease control and eradication programs, marker vaccines are also limited by multiple dose administration requiring and lower efficiency, if compared to live attenuated vaccines (M. D. Baron et al. 2016).

Poxvirus vectored vaccines. Because of its large dsDNA genome containing several replaceable non-essential genes and being easily suitable for genetic manipulations, the first recombinant viral vector being exploited as a marker vaccine was the Vaccinia virus (VV) belonging to the *Poxviridae* Family. Specifically a recombinant Vaccinia-rabies vaccine has been developed, eliciting an effective protective immunity in vaccinated wild animals and being resistant to the field extreme temperatures, thus being nowadays commonly used for rabies control in both Europe and USA. Moreover recombinant Vaccinia-rinderpest vaccines, expressing both RPV F and H antigens, have also been developed, but since these vaccines were based on the Western Reserve (WR) Vaccinia strain, they were not considered safe enough for being licensed in case of smallpox absence. Vaccinator's safety represents, indeed, one of the most concerning issues of using a recombinant vaccinia virus-based vaccines. In addition, probably due to their relatively high virulence, vaccinia virus-based vaccines have been seen to cause severe lesions at animal inoculation sites. To overcome

these issues, two other more sufficiently attenuated poxviruses strains, the LC16mO and the Wyeth capripox viruses, causing pox in sheep and goats, have subsequently been adopted. The established capripox virus-based vaccines have been employied in both rinderpest and PPR control campaigns. The recombinant capripox virus-based vaccine showed different advantages, such as over three years long protection in cattle and cross-protection against the LumpySkin Disease (LSD) in cattle and the PPR disease in sheep and goats. However, pre-existing anti LSD virus antibodies interfering with the vaccine intake, represent a disadvantage for its application. A recombinant capripox vectored vaccine has then been developed against the endemic PPR, being relatively thermostable and giving also cross-protection against sheep and goats pox. F and H PPRV glycoproteins coding sequences have separately been cloned into the attenuated capripox virus genome, and the resulting recombinant viral vector was effective in inducing immunity against both PPR and capripox in inoculated animals. However, probably due to a preexisting immune protection against the capripox vector itself, this vaccine is not able to fully protect vaccinated animals from PPRV challenge (M. D. Baron et al. 2016; Kumar et al. 2017).

Adenovirus vectored vaccines. Since many studies highlight their efficiency as vaccine delivery vehicles, adenoviruses have also been exployited for PPRV DIVA vaccines development. Adenoviruses keep genetic stability over multiple culture passages and can be easily expanded, producing high virus quantities with low expense. The adenovirus type 5 (Ad5) is the most tested human virus as a vector. Since small ruminants lacks of peexisting immunity against it, Ad5 is likely to be a good vector for animal vaccination. Moreover adenoviruses show a better thermotolerance compared to morbilliviruses, and to improve their preparations thermostability and facilitate their storage and delivery to hot and humid climate areas, different methods have been developed. The so generated PPRV-F and H recombinant Ad5-based vaccines, were able to induce both specific humoral and cell-mediated immune responses in small ruminants, protecting them from PPRV infection. The Ad-H and Ad-F combination showed to be better protective than the Ad-H or Ad-F delivered alone. Moreover, a PPRV-H recombinant canine adenovirus-based vaccine, inducing anti-PPRV-H neutralizing antibodies, has also been developed (M. D. Baron et al. 2016; Kumar et al. 2017).

Morbilliviruses Cross Protection

It is of interest to explore whether there can be cross protection among morbilliviruses, since it has been well observed and demonstrated that sera from morbillivirus-infected animals can cross react to other morbilliviruses. For example, the increase of CDV-caused disease outbreacks in primate, but not human populations, might suggest that MV circulation in humans might have cross-protected them from the CDV disease. However, cross protection is strictly related to virus strain and animal host biology, thus it is not always a reciprocal phenomenon between all morbilliviruses. In fact, as an example, it has been seen that CDV is able to cross protect cattle against RPV, but this last one does not protect cattle back from CDV, since it can only provide cross protection against CDV in dogs and partially in ferrets. The RPV-PPRV cross-relationship has also been verified. It has been experimentally observed that antibodies from PPRV vaccinated goats, can *in vitro* neutralize RPV in cell culture, even though with a lower titer compared to PPRV neutralization, and that, reciprocally, antibodies from RPV vaccinated goats, well protect from rinderpest, but show very low PPRV neutralization. Likewise, Holzer et al. (Holzer et al. 2016) have recently proved that PPRV vaccine elicits both humoral and cell-mediated protective immune responses in cattle, but failed the RPV challenge. However, in accondance to Mornet et al. (Mornet, Orue, e Gilbert 1956) earlier studies, where crude materials from PPR infected animals were proved to protect cattle against RPV, they also observed that, only when both Nigeria/75 and Sungri/96 PPRV vaccine strains were independently inoculated in cattle along with a virulent PPRV strain, there was cross-protection against the virulent RPV.

To date, the exact morbilliviruses cross-protection mechanism remains elusive. It seems to be mainly mediated by cellular immunity rather than cross-reacting antibodies activities. When antibodies-mediated cross-protection occurs, it is not due to neutralizing antibodies presence but it is rather due to fixing complement cross-reactive antibodies, thus observing infected cells death but no prevention from further cell culture infections. Since it has been observed that antibodies against RPV-F do not directly neutralize the virus, but rather only in presence of complement, while anti-RPV-H antibodies directly neutrlize it, this morbilliviruses antibodies-mediated cross-protection might be due to fusion glycoprotein conserved epitopes. Likewise, Gould et al. (Gould et al. 1981) experiments of indirect immunofluorescence titrations, using prepared monospecific antisera against either the MV haemagglutinin (anti-HA), haemolysin (anti-HL), or ribonucleoprotein (anti-RNP), showed cross-reaction with CVD surface glycoproteins. However, no CVD neutralization, with any of the monospecific sera, neither singoularly or combinally tested, has been dectected. These

results might highlight an antigenic variation occurrance between different CVD strains, and the existance of unique antigenic determinants, both for MV and CDV.

Thus, it has been hypothetized that all these cross-protection differences among morbillivirus prototypes might be due to virus strain intrinsic abilities of stimulating immune responses and, since their RNA genomes are highly susceptible to nucleotide/s variations, to the nature of vaccine random attenuating mutations outcoming after several culture passages. Since the attenuating outcoming mutations are random and not characterized, it is possible that they might affect viral immunogenicity and, therefore, host immune responses efficiency (Kumar et al. 2017).

AIM OF WORK

The vectorialized recombinant vaccine generated and experimented in this PhD project aims to satisfy the guide lines provided by the Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (OIE) for Peste des Petit Ruminants disease (PPR) Global Control and Eradication Strategy (PPR GCES) from endemic countries. Investing in PPR eradication significantly meets the objectives of Sustainable Development Goals (SDGs), contributing to food security and reducing vulnerable pastoral and rural communities poverty. More than half of the African population economy, which is primarily based on small-scale farming, still presents high rates of persistent poverty and food insecurity. Most of these farming households are engaged in ruminants livestock, that represent the major source of economy activity and high-protein nutrition for the population. Multiple factors, including infectious diseases, drastically constrain pastoralist households livestock productivity (Marsh et al. 2016).

Mortality due to infectious diseases could however be efficiently prevented by vaccination, as it has been proved by the FAO Global Rinderpest Eradication Programme (GREP), based on widespread campaigns for cattle and buffaloes that allowed to successfully eradicate the Rinderpest (RP) disease in June 2011. PPR disease is a highly contagious disease of wild and domestic small ruminants occurring throughout Africa, the Middle East, Turkey, West and South Asia, and China, caused by the Peste des Petit Ruminants virus (PPRV), a closely related morbillivirus to the Rinderpest virus (RPV), the etiological agent of the RP disease. PPR causes RP similar clinical signs, thus it has been initially widely confused with the RP disease, and has been just first reported in 1942 in Africa. Since then, the PPR disease has highly spreaded over several countries, infecting hundred of millions of small ruminants, causing serious socio-economic losses and damages to affected small-scale farmers and pastoralists, with an estimated annual global impact of US$ 1.4 -2.1 billion. As well as it has been done against the RP disease, investing in a PPR eradication program would significantly contribute to the economy and food security of the world's most vulnerable pastoral and rural communities. Despite protective anti-PPR vaccines based on PPRV live attenuated forms are currently available on the market, they are not suitable for serum surveillance, which is indispensable for an eradication campaign.

Thus, the objective of this experimental work has been the generation of a new generation vaccine based on a recombinant Bovine herpesvirus type 4 (BoHV-4) against PPRV, delivering PPRV Hemagglutinin (H) surface glycoprotein (BoHV-4-A-PPRV-H-ΔTK), that

covers efficiency and protection vaccination requirements with the possibility of animals serum surveillance.

BoHV-4 is a γ-herpesvirus with feasible antigen delivery vector characteristics, since it has been shown capable of replicating into both murine and ruminant animal models with no evidences of pathogenicity or growth transformation activities. The recombinant BoHV-4-A-PPRV-H-ΔTK has been efficiently generated through the heat inducible SW102 *E coli* strain recombineering system and tested as a potential DIVA vaccination platform in immunocompetent C57BL/6 murine animal models. The experimentally BoHV-4-A-PPRV-H-ΔTK-immunized mice have been tested for anti-PPRV-H specific T cell and neutralizing antibody immune responses. These preliminary experimental data on mice immunization will serve as a first test of BoHV-4-A-PPRV-H-ΔTK-based vaccine potential efficacy. Future prospectives will be to subsequently test this DIVA vaccine on PPRV natural host in order to assess its potential applicability on field.

MATERIALS AND METHODS

Cells and viruses

In this PhD thesis project, Human Embryo Kidney 293T (HEK 293T)- (ATCC: CRL-11268), Bovine Embryo Kidney (BEK)-, provided from Dr. M.Ferrari, Istituto Zooprofilattico Sperimentale, Brescia, Italy (BS CL-94), and BEK *cre*,- expressing the *cre* recombinase enzyme, cell lines (G. Donofrio et al. 2008), have been cultured in Eagle's Minimal Essential Medium (EMEM, Gibco) completed with 10% heat inactivated Fetal Bovine Serum (FBS), 2 mM L-glutamine (Gibco), 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (SIGMA), and 0.25 µg/ml amphotericin B (Gibco). The cultured cells have been incubated at 37°C in a 95% air humidified atmosphere incubator with 5% CO_2. Vero Dog-SLAM (VDS)-, stably expressing canine SLAM receptor (Seki et al. 2003), and murine RMA-s- (RRID:CVCL_2180), expressing low Major Histocompatibility Class I (MHC I) molecules on their surface (Esquivel, Yewdell, e Bennink 1992), cell lines were kindly provided from Dr. Parida (IAH, Pirbright, UK) and Dr. McArdle (The Nottingham Trent University, UK), respectively. VDS cell line was grown in Dulbecco modified Eagle medium (DMEM) supplemented with 7% heat-inactivated FBS, 0.15% sodium carbonate, and 0.4 mg/ml of G418 (José M. Rojas, Moreno, et al. 2014), while murine RMA-s cell line was cultured in T cell Roswell Park Memorial Institute (RPMI) media added with 10% Fetal Calf Serum (FCS), 4 mM L-glutamine, 10 mM N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid (HEPES),1% 100X non-essential amino-acids,1 mM sodium pyruvate,100 U/ml penicillin-100 µg/ml streptomycin + 50 nM β-mercaptoethanol) (J.-M. Rojas et al. 2011).

Constructs generation

The synthetic PPRV-H open reading frame has been first amplified from linearized pGEM-T Easy-PPRV-H (Eurofins) vector template by PCR using NheI-PPRV-H sense (5′-ccccgctagcccaccatgtccgcacaaagggaaagg-3′) and Phos-PPRV-H antisense (5′-agactggattacatgttacctc-3′) primers pair to insert the NheI restriction site at the ORF 5′ terminus and a phosphate group at the ORF 3′ terminus. The so generated PPRV-H amplicon has then been cloned into the NheI/SalI blunt cut pIgK-E_2BVDV$_3$-gD$_{106}$ intermediate shuttle vector (A. Capocefalo et al. 2010) in order to generate the pIgK-PPRV-H-gD$_{106}$ vector,

expressing the PPRV-H antigen gD_{106} tagged at the carboxy terminus. The gD_{106} tagged fragment has then been excised from the intermediate plasmid cutting with NheI and BamHI blunt restriction enzymes and subsequently cloned into the NheI/SmaI cut $pINT_2$-EGFP vector(Clontech) to generate the $pINT_2$-PPRV-H-gD_{106} final shuttle vector, necessary for BAC-BoHV-4 homologous recombination (Macchi et al. 2018).

Transient transfection

Transfection assays have been performed on HEK 293T cells. Cells were seeded into a six wells plate at 3×10^5 cells/well density, and incubated at 37°C in a 95% air humidified atmosphere incubator with 5% CO_2.. Culture medium has been removed after cell monolayer has reached sub-confluence, and cells have been transfected with pIgK-PPRV-H-gD_{106}, $pINT_2$-PPRV-H-gD_{106}, and pEGFP-C1 (Clontech) ,as negative control,vectors using Polyethylenimine (PEI) transfection reagent (Polysciences, Inc.). 3 µg of vector DNA have been mixed with 7.5 µg PEI (1 mg/ml concentrated) at the ratio 1:2.5 DNA:PEI, in 200 µl of high glucose Dulbecco's Modified Essential Medium (DMEM) (Euroclone) with no serum added, and 15' incubated at Room Temperature (RT). 800 µl of high glucose DMEM with no serum have then been added to the tranfection solution and transferred to the cell monolayer and 6 hours incubated at 37°C in a 95% air humidified atmosphere incubator with 5% CO_2.. After incubation, the transfection solution has been replaced with fresh EMEM, completed with 10% FBS, 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B, and 24 hours incubated at 37°C in a 95% air humidified atmosphere incubator with 5% CO_2..

Western immunoblotting

Protein cell extracts from pIgK-PPRV-H-gD_{106}, $pINT_2$-PPRV-H-gD_{106}, and pEGFP-C1-transfected HEK 293 T cells and BoHV-4-A-PPRV-H-ΔTK-infected BEK cells have been collected from a six-well confluent plate and 25-cm^2 confluent flasks, respectively, by using 100 µl of cell extraction buffer (composed of 50 mM Tris–HCl at pH 7.4, 250 mM NaCl, 2mM EDTA and 1% NP-40; at pH 8), added with protease inhibitor cocktail-EDTA-free (BioVision) 1:200 and PhenylMethylSulfonyl Fluoride (PMSF) 1:100. Protein cell extracts have been analyzed on a 10% SDS-PAGE gel electrophoresis, using a 1X Running buffer (3% Tris 250mM, 20.6% Glycine, 1% SDS, in 1L of ddH_2O) and transferred on a

Polyvinylidene Difluoride (PVDF) membrane by electroblotting, using a 1X Transfer buffer (3.03g Tris, 14.41g Glycine, in 1L of ddH_2O). The resulting membranes have been 1 hour blocked in 5% skim milk in 1X TBST buffer solution (1M TrisHCl, 1M NaCl, 5ml Tween20, in 1L of ddH_2O) , three times 5 min washed with 1X TBST, 1 hour incubated with a 1:10,000 diluted primary bovine anti gD_{106} monoclonal antibody (clone 1B8-F11; VRMD, Inc., Pullman, WA, USA), three times 5 min washed with 1X TBST solution, and finally 1 hour probed with a 1:10,000 diluted horseradish peroxidase-labeled anti-mouse immunoglobulin (SIGMA). Protein bands have then been visualized by enhanced chemiluminescence: the surface membrane has been covered with Chemiluminescent substrate (Clarity Max ECL Western Blotting Substrates, Biorad) drops, and the membrane placed between two acetate sheets. A damp lab tissue has been used to eliminate any bubbles present under the sheet and create a liquid seal around the membrane. Protein bands detection has then been obtained by 2-60 seconds exposing the membrane into the ChemiDoc XRS (Biorad).

BAC-BoHV-4-A recombineering and selection

The heat inducible BAC Recombineering assay has been performed as designed by Warming et al. (Warming et al. 2005), applying some modifications. 500µl of a 32°C Over Night (ON) shacking culture of SW102 *Escherichia coli* (*E. coli*) strain containing the pBAC-BoHV-4-A DNA plasmid, have been diluited into 25ml of Luria Bertani (LB) medium with 12,5µg/ml Chloramphenicol (SIGMA) selection and have been grown in a 32°C shacking incubator until it reached OD_{600} 600nm. The culture has then been divided into two conical flasks, 12ml each, and 42°C heat induced for 15 min in a shaking water bath. After the heat induction, bacteria have been briefly cooled in ice and subsequently transferred into two 50ml falcons where they have been 5 min 6000rpm pelletted at 4°C. After supernatants removal, pellets have been twice gently resuspended into 10ml of ice-cold ddH_2O and 5 min 6000rpm re-pelleted at 4°C. After supernatants removal, each pellets have been resuspended into almost 100µl of ddH_2O and electroporated with the electrophoresis gel-purified, *PvuI* linearized, pTK-CMV-PPRV-H-TK expression cassette, in a 0.1cm couvette at 25µF, 2.5KV and 201Ω. Right after electroporation, bacteria have been recovered into 10ml of LB for 4 and a half hour in a shaking 32°C incubator. After recovery, 1ml of bacteria have been 2min 6000rpm pelletted at RT in an eppendorf tube and washed twice in 1ml of sterile 1X M9 salt solution (6g/L Na_2HPO_4, 3g/L KH_2PO_4, 1g/L NH_4Cl and 0.5g/L NaCl; SIGMA). M9 washes serves for rich media residues removal from bacteria prior selection on minimal medium

plates. After the second M9 wash, supernatant has been removed and pellet resuspended in 1ml of sterile 1X M9 salt solution and plated at serial diluitions (1:10, 1:100 and 1:1000) on M63 minimal medium plates (15g/L agar (DIFCO, BD Bioscences), 0.2% D-galactose (SIGMA), 1mg/L D-biotin (SIGMA), 45mg/L L-leucine (SIGMA) and 50mg/L Kanamycin (SIGMA)), 250µl each. Plates have been 3-5 days 32°C incubated and grown colonies have been picked up and both negatively and positively selected in 500µl with Kanamycin and Cloramphenicol respectively. Only the resulting Kanamycin negative and Chloramphenicol positive colonies have been kept and grown ON in 5ml of LB with 12.5 mg/ml Chloramphenicol. BAC-DNAs have been purified and analyzed through *HindIII* restriction enzyme digestion. DNA has then been separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and visualized through UV light. Original detailed recombineering protocols are also documented at the "https://redrecombineering.ncifcrf.gov/" recombineering website.

Southern blotting

To further confirm the positively recombineered clones, a Southern Blotting assay based on a H sequence-spanning probe has been performed. The *HindIII* digested BAC-DNAs ON separated on a 1% electrophoresis agarose gel, have been capillary transferred to a positively charged nylon membrane (ROCHE) and cross-linked by UV irradiation, using standard procedures (G. Donofrio et al. 2008). After the ON electrophoresis, the agarose gel has been first 15 min washed with 250ml of depurination solution (0.25M HCl), untill the loading buffer dye turned yellow, then 15min washed with 250ml of denaturation solution (1.5 M NaCl, 0.5 M NaOH), and finally 20min washed with 250 ml of neutralization solution (1.5 M NaCl, 0.5 M Tris at pH 7.5, 1mM EDTA) ,all washing in a shaking water bath. After the washing the agarose gel has been ON brought into contact with the nylon membrane. The capillarity DNA transfer from the gel to the membrane is mediated by the draining of SSC 20X buffer (3 M NaCl, 0.3 M sodium citrate). After the covalently DNA crosslinking to the membrane in an UV oven, the membrane has been 1 h pre-hybridized into 50 ml of hybridization solution (7% SDS, 0.5 M phosphate, pH 7.2) in a 65°C rotating hybridization oven (Techna Instruments). The digoxigenin non-isotopic labeled H probe has been generated by PCR, using the *NheI*-PPRV-H sense (5′-ccccgctagcccaccatgtccgcacaaagggaaagg -3′) and Phos-PPRV-H antisense (5′-

agactggattacatgttacctc -3′) primers. The PCR amplification reaction has been carried out in 50 µl of final volume, containing 10 mmol Tris–hydrochloride at pH 8.3, 5% Dimethyl Sulfoxide (DMSO), 0.2 mmol deoxynucleotide triphosphates, 2.5 mM $MgSO_4$, 50 mM KCl, and 0.25 µM of each primer. 100ng of DNA have been amplified over 35 cycles, each consisting of 1 min denaturation at 94°C, 1 min primer annealing at 60°C, and 2 mins chain elongation with 1U of Taq DNA polymerase (Fermentas) in addition to 1 µl of Digoxigenin-11-dUTP, alkali-labile (Roche Life Science) at 72°C. H probe 5µl have been added to ddH_2O 500µl and 5 min denaturated in boiling water, then 2 min cooled down in ice. The H denaturated probe has then been added to the hybridization solution, and the membrane ON hybridized at 65°C in a rotating hybridization oven (Techna Instruments). After hybridization, the membrane has been washed extensively twice for 15 minutes each in Washing Solution 1 (0.5X SSC, 0.1% SDS) and twice for 15 minutes each in Washing Solution 2 (40mM PO_4 pH7.2 and 0.05%SDS) at 65°C. After a quick equilibration of the membrane in washing buffer (100mM maleic acid, 150mM NaCl, pH7.5 and 0.3% tween 20) at room temperature for 1 minute, the membrane has been blocked in 100 ml of blocking solution (100mM maleic acid, 150mM NaCl, pH7.5 and 1% of Blocking reagent from Roche [11096176001] by gently agitation for at least 30 minutes. Antidigoxigenin Fab fragment (150U/200µl (Roche)), diluted 1:15,000 in 50 ml of blocking solution, has been finally applied to the membrane, and detection has been performed following two extensive 15 minutes washes in washing buffer and a 2 min membrane equilibration at RT, in detection buffer (100mMTris/HCl, 1mM EDTA, pH9.5) (Gaetano Donofrio, Franceschi, Capocefalo, Taddei, et al. 2009). The surface membrane has been covered with Chemiluminescent substrate (Roche) drops, and the membrane placed between two acetate sheets. A damp lab tissue has been used to eliminate any bubbles present under the sheet and create a liquid seal around the membrane. Signal detection has then been obtained by exposing the membrane to a X-ray film. Exposure time has been adjusted on the signal intensity.

Cell culture electroporation and recombinant virus reconstitution

BEK and BEK*cre* cells have been maintained as a monolayer with complete DMEM growth medium, added with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. When cells have reached the 70–90% confluence, they have been splitted in a fresh culture flask (i.e., every 3–5 days) and incubated at 37°C in a 5% CO_2 humidified

atmosphere. 5 µg of pBAC-BoHV-4-A-PPRV-H-ΔTK have been electroporated into BEK and BEK*cre* cells harvested from a confluent 25-cm^2 flask, in 600 µl DMEM with no serum, using a 4-mm gap cuvettes and the Equibio Apparatus, 270 V, 960 mF. Electroporated cells have then been re-plated into a 25-cm^2 flask, and after 24 hours the medium has been replaced with fresh one. At two days post-electroporation, when cells have reached the confluence, they have been 1:2 splitted and left to grow until cytopathic effect (CPE) appearance.

Viruses and viral replication

BoHV-4-A-PPRV-H-ΔTK and BoHV-4-A have been propagated and amplyfied by infecting BEK cells confluent monolayers at a Multiplicity Of Infection (MOI) of 0.5 Tissue Culture Infectious Doses 50 ($TCID_{50}$) per cell and 2 hours maintained in 2% FBS medium. The medium has then been removed and replaced with fresh 10% FBS EMEM. At almost 72 hours post-infection, when most of the cell monolayer presented CPE, the virus has been prepared by three times freezing at -80°C and thawing the infected cells and pelleting the virions through a 30% sucrose cushion, as previously described (Gaetano Donofrio, Cavaggioni, et al. 2006). Cell debris have been removed by low-speed centrifugation, and virions were pelleted through a 3 ml of 30% sucrose cushion in PBS, using a Beckman SW41 rotor at 35,000 rpm for 90 min at 4°C. Virus pellets have then been resuspended in cold EMEM without FBS. $TCID_{50}$ have been determined on BEK cells by limiting dilution.

Viral growth curves

BEK cells have been BoHV-4-A and BoHV-4-A-PPRV-H-ΔTK infected at a M.O.I. of 0.1 $TCID_{50}$/cell and 4 hours incubated at 37°C in a 5% CO_2 humidified atmosphere. Infected cells were washed with serum-free EMEM and then overlaid with 10% FBS EMEM added with 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml Amphotericin B. Supernatants from infected cultures, containing the viruses, have been harvested 24, 48, 72, and 96 hours post-infection, and the amount of infectious virus has been determined by limiting dilution on BEK cells. Viral titer differences between each time point represent the averages of triplicate measurements ± mean standard errors ($p > 0.05$ for all time points as measured by Student's *t*-test).

Animals and immunizations

Seven- to eight-weeks old female C57BL/6 mice (Harlan) have been intraperitoneally (ip) inoculated and boosted 21 days after, with PBS (group 1; $n = 10$), 10^6 $TCID_{50}$/ml of BoHV-4-A (group 2; $n = 10$) and 10^6 $TCID_{50}$/ml of BoHV-4-A-PPRV-H-ΔTK (group 3; $n = 10$). Animals have been bled at 14, 28, and 36 days post first immunization. Five animals per group have been sacrificed at day 7 post-booster in order to perform T cell response experiments. All animal experiments have been performed in a disease-secure isolation facility (BSL3) at the CISA (CISA-INIA, Madrid) in strict accordance with the Code for Methods and Welfare Considerations in Behavioral Research with Animals recommendations (Directive 867609EC; RD1201/2005).

Flow cytometry intracellular cytokine staining assays

As previously described (J.-M. Rojas et al. 2011), splenocytes from inoculated C57BL/6 mice have been obtained by mechanical disruption and cultured in T cell media (RPMI added with 10% FCS, 4 mM L-glutamine, 10 mM HEPES, 1% 100X non-essential amino-acids, 1 mM sodium pyruvate, 100 U/ml penicillin/100 µg/ml streptomycin, 50 nM β-mercaptoethanol). IFN-γ ELISPOT assays have been performed according to the manufacturer protocol (Diaclone, France). As negative control, cells have been cultured either with no stimuli, DMSO (same volume as the one added with peptide), or MC57 cell line lysate (used in the virus preparation). All cultures have been performed in triplicates and the results were presented as average spots number for each mouse. Assays have been considered valid only when IFN-γ spot counts in 2×10^5 cells control wells were below 20, and standard deviations in positive average wells below 10%. 0.5 µg/ml Concanavalin-A activated Splenocytes, has always been included as positive control in order to validate the ELISPOT assay. To measure responses to PPRV, splenocytes have been ON cultured with Binary ethylenimine (BEI)-inactivated Nig'75 PPRV strain (José M Rojas, Peña, et al. 2014). To assess murine T cell specific anti-PPRV-H, , splenocytes have been 1 week *in vitro* expanded with 10 µg/ml of PPRV-H H5 (H(551-559) YFYPVRLNF) and H9 (H(427-441) ITSVFGPLIPHLSGM) epitopes (José Manuel Rojas et al. 2017) before measuring IFN-γ responses. For intracellular IFN-γ production measurements, cells have been cultured at 10^6 cells per well in presence of different ON stimuli (peptide or PPRV) before adding 10 µg/ml brefeldin-A (SIGMA) for the last 5 hours of incubation. 20 ng/ml phorbol myristyl acetate (SIGMA) and 1 µg/ml ionomycin (SIGMA) stimulation have been used as positive

controls for IFN-γ production. Vehicle (DMSO)-stimulated (no peptide) or irrelevant peptides (lymphocytic choriomeningitis virus gp33-41 peptide (KAVYNFATC)) have been used as negative controls. No differences in background IFN-γ production has been detected between these negative control groups. After stimulation, cells have been anti-mouse CD4-FITC and anti-mouse CD8-PerCP antibodies (BDpharmingen) stained. Cells have been fixed and permeabilized in PBS added with 4% paraformaldehyde and 0.1% saponin (wt/vol). Cells have then been anti-mouse IFN-γ-PE (BD pharmingen) stained and acquired using a FACSCalibur flow cytometer (Becton Dickinson). Gating strategy has been performed as previously described (José Manuel Rojas et al. 2017). Gating for IFN-γ positive events has been set by using isotype and fluorescence minus one channel controls. The resulting data have been analyzed with the FlowJo software (TreeStar Inc.).

Flow cytometry cytotoxicity assays

BoHV-4-A-PPRV-H-ΔTK immunized mice splenocytes have been 1 week *in vitro* expanded with H5 peptide. H5 stimulated splenocytes have been used as effector cells in flow cytometry cytotoxicity assay. RMA/s target cells have been PKH67 green fluorescent linker labeled (José M. Rojas et al. 2016) and pulsed with relevant peptide. Vehicle-pulsed (no peptide) RMA/s cells have been used as negative control. Effector cells and target cells have then been 4 hours incubated at 37°C in 96 U-bottom well plates. Cells have subsequently been transferred to FACS tubes, dead cells labeled with 2 µg/ml propidium iodide (PI), and samples immediately analyzed by flow cytometry. Target cells have been gated on bright FL1+ cells. Maximum cell death (target cells in PBS added with 0.2% saponin) and spontaneous cell death have been used as positive controls in all experiments. The specific target cell lysis percentage has been calculated based on the formula: % specific lysis = 100 × (% PI+ target – % spontaneous death)/(% maximum death − % spontaneous death).

PPRV neutralization assays

Serum samples have been 30 min inactivated at 56°C and tested for neutralizing antibodies presence as previously described (Barrett et al. 1989). Briefly, Nigeria 75/1 PPRV strain stock has been 1 hour incubated with serial dilutions of inactivated sheep serum at RT, in triplicate. VDS cells have been added at the concentration of 1.5×10^5 cells/ml to each well

and 7 days incubated, then fixed with 2% formaldehyde and cells visualized by crystal violet staining. Wells without virus served as negative controls. The plates have been 7 days monitored for PPRV CPE formation. The VNT titer has been defined as the highest serum dilution that inhibited 50% of the CPE. Sera with VNT 1:10 titers have been considered negative.

Statistical analysis

Power analysis (Charan e Kantharia 2013) has been used to determine treatment group size for T cell responses and PPRV seroneutralization assessment. Statistical analysis have been performed using Prism 5.0 software (Graphpad Software Inc., USA) and Mann–Whitney test has been used to compare IFN-γ production in CD4+ and CD8+ T cells. Significance levels are $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

Recombinant BoHV-4-A-PPRV-H-ΔTK generation

Since the PPRV-H surface glycoprotein represents the virus immuno dominant antigen, potentially capable of inducing a protective immune response, it has been chosen as candidate for BoHV-4 recombination. The PPRV-H protein expression has been optimized by cloning its sequence under the Immediate Early CMV gene promoter transcriptional control into the CMV-PPRV-HgD_{106} - gD_{106} tagged expression cassette. The recombinant BoHV-4 has been generated via heat inducible homologous recombination in an engineered *E.coli SW102* strain containing the pBAC-BoHV-4-A-KanaGalK-ΔTK plasmid. Recombination occurred between the BoHV-4 TK homologous sequence of the $pINT_2$-CMV-TK-PPRV-HgD_{106}-TK shuttle vector and the pBAC-BoHV-4-A-KanaGalK-ΔTK plasmid, replacing the Kana/GalK cassette with the CMV-PPRV-HgD_{106} expression cassette of interest, and generating the pBAC-BoHV-4-A-PPRV-H-ΔTK (*Fig.26*).

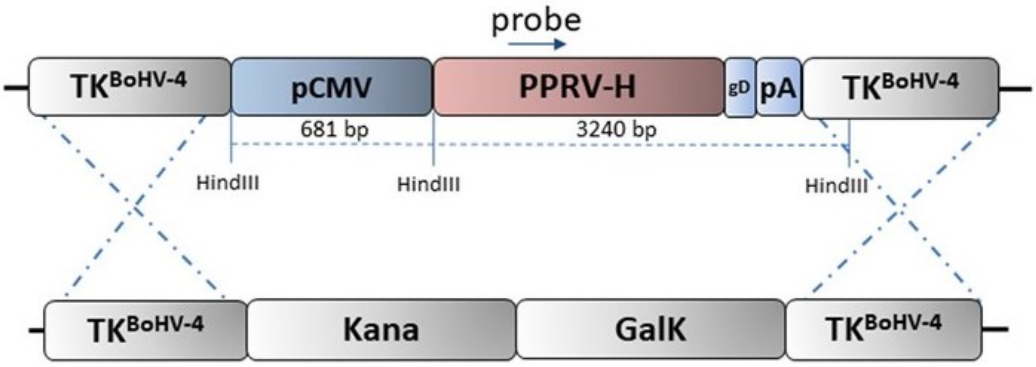

Figure 26. **Diagram (not to scale) of the heat-inducible homologous recombination between the Kana/GalK cassette and the CMV-PPRV-HgD_{106} expression cassette of interest, flanked by BoHV-4-TK homologous sequences, occurring in *E.coli SW102* strain.**

The 2-deoxy-galactose resistant clones have been first screened by *HindIII* restriction enzyme analysis on electrophoresis gel. The positively recombined pBAC-BoHV-4-A-PPRV-H-ΔTK viral genomes showed a 3240bp band, replacing the 2650bp band corrisponding to the Kana/GalK cassette of the un-retargeted pBAC-BoHV-4-A-TK-KanaGalK-TK control virus. The presence of the PPRV-H ORF into recombinant viral

genomes has subsequently been confirmed by Southern Blotting assay using a PPRV-H ORF specific probe (*Fig.27*). The clone stability has been assessed over 20 growth passages.

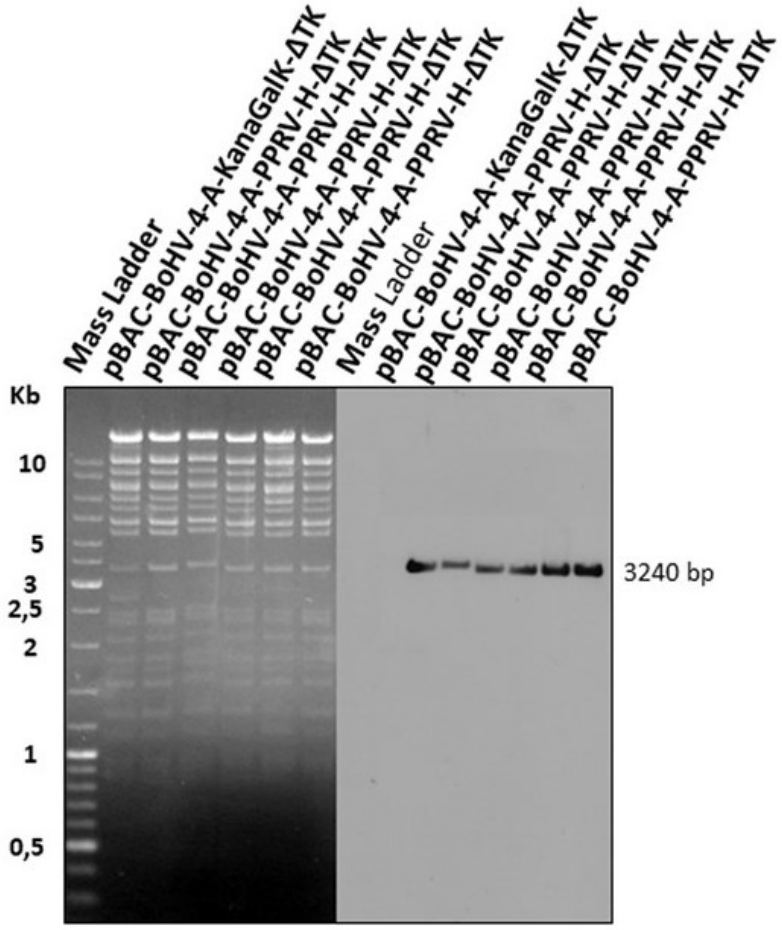

Figure 27. **Positive recombinant pBAC-BoHV-4-A-PPRV-H-ΔTK clones *HindIII* restriction enzyme analysis on agar gel electrophoresis (on the left) and PPRV-H ORF-based Southern Blotting assay (on the right).**

The 2.650 bp Kana/GalK cassette band of the un-retargeted pBAC-BoHV-4-A-TK-KanaGalK-TK has been replaced by the 3.240 bp PPRV-H band of the positively recombined pBAC-BoHV-4-A-PPRV-H-ΔTK clones.

Viable recombinant BoHV-4-A-PPRV-H-ΔTK infectious viral particles have then been obtained by electroporating the pBAC-BoHV-4-A-PPRV-H-ΔTK plasmid into BEK and BEK*cre* cells (*Fig.28*). Both cell monolayers showed viable reconstituited recombinant BoHV-4 particles plaques. BEK cell monolayer showed the typical green fluorescence due to the BAC/GFP cassette. BEK*cre* cells, containing the *Cre* recombinase enzyme, allowed

the excision of the BAC/GFP cassette from the recombinant BoHV-4-A-PPRV-H-ΔTK genome, determining the loss of green fluorescent plaques on the cell monolayer.

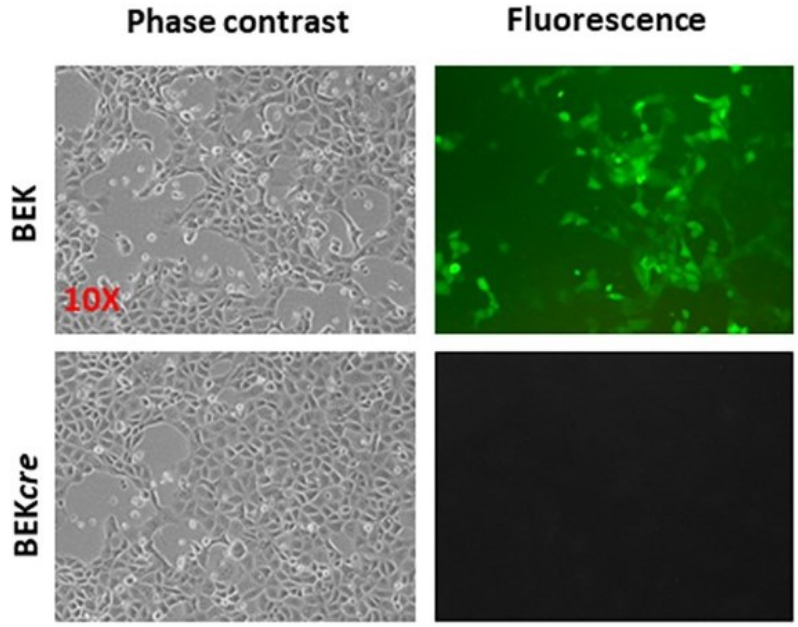

***Figure 28*. 10X magnification of contrast phase and fluorescent microscopic images of BEK and BEK*cre* cells electroporated with pBAC-BoHV-4-A-PPRV-H-ΔTK plasmid.**

Both cell monolayers show viable reconstituited recombinant BoHV-4 particles plaques. BEK cell monolayer shows the typical green fluorescence due to the BAC/GFP cassette, while the *Cre* recombinase contained into the BEKcre cells determines the excision of the BAC/GFP cassette from the recombinant BoHV-4 genome and the loss of fluorescent viral plaques on the cell monolayer.

The replication kinetics up to 96 hours post-infection of the so obtained recombinant BoHV-4-A-PPRV-H-ΔTK infectious particles grown on BEK cells showed no replication defects compared to the BoHV-4-A parental strain isolate (*Fig.29*).

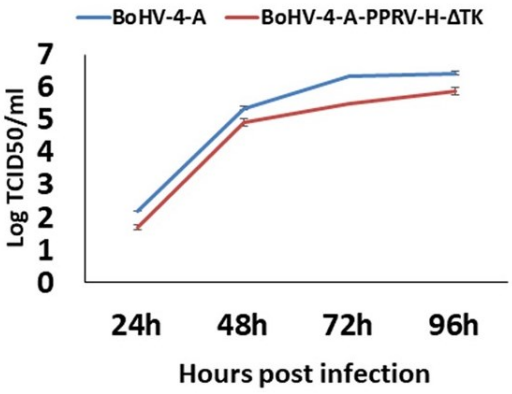

Figure 29. **BoHV-4-A-PPRV-H-ΔTK replication kinetics time points up to 96 hours post-infection grown on BEK cell monolayer, compared to the BoHV-4-A parental strain isolate.**
The graphed data represent the mean ± standard errors of triplicate virus replication kinetic measurements for each time point ($P > 0.05$) measured by Student's t-test.

Moreover, the recombinant BoHV-4-A-PPRV-H-ΔTK-infected BEK cells protein extract has been evaluated by Western immunoblotting using a primary antibody directed against the PPRV-H gD106 tag (*Fig.30*). The resulting data, compared to the BoHV-4-A-infected BEK cells negative control protein extract, showed that PPRV-H protein is well expressed by the recombinant BoHV-4-A-PPRV-H-ΔTK.

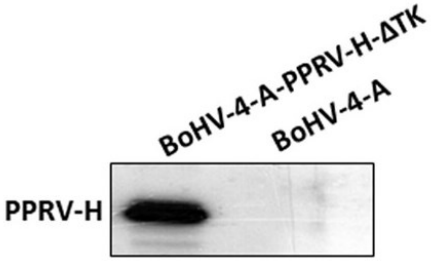

Figure 30. **Western immunoblotting of infected BEK cells protein extracts.**
Evaluation of PPRV-H protein expression from BoHV-4-A-PPRV-H-ΔTK (left lane) and BoHV-4-A (right lane) -infected BEK cells protein extracts by Western immunoblotting. The BoHV-4-A-infected BEK cells protein extract serves as negative control.

Anti-PPRV T Cell response induced by BoHV-4-A-PPRV-H-ΔTK immunization

In order to measure the induction of the anti-PPRV T cell immune response, 7 days after booster immunization, splenocytes have been extracted from the BoHV-4-A-PPRV-H-ΔTK vaccinated C57BL/6 mice, ON stimulated with the inactivated PPRV Nig'75 strain (BEI-PPRV Nig'75), and tested by flow cytometry for T cells IFN-γ production against PPRV, using an intracellular staining. As a result, only the BoHV-4-A-PPRV-H-ΔTK immunized mice CD4+ T cells have produced IFN-γ in response to inactivated PPRV Nig'75 strain stimulation, while no specific anti-PPRV IFN-γ production have been detected from both PBS- and BoHV-4-A- immunized mice CD4+ T cells. A similar result has been observed for induced CD8+ T cells, as the IFN-γ production has been detected only from BoHV-4-A-PPRV-H-ΔTK immunized mice CD8+ T cells and not from either PBS- or BoHV-4-A-immunized animals CD8+ T cells. These data demonstrated that BoHV-4-A-PPRV-H-ΔTK immunization in C57BL/6 mice elicited both CD4+ and CD8+ T cell anti-PPRV specific immune responses (*Fig.31*).

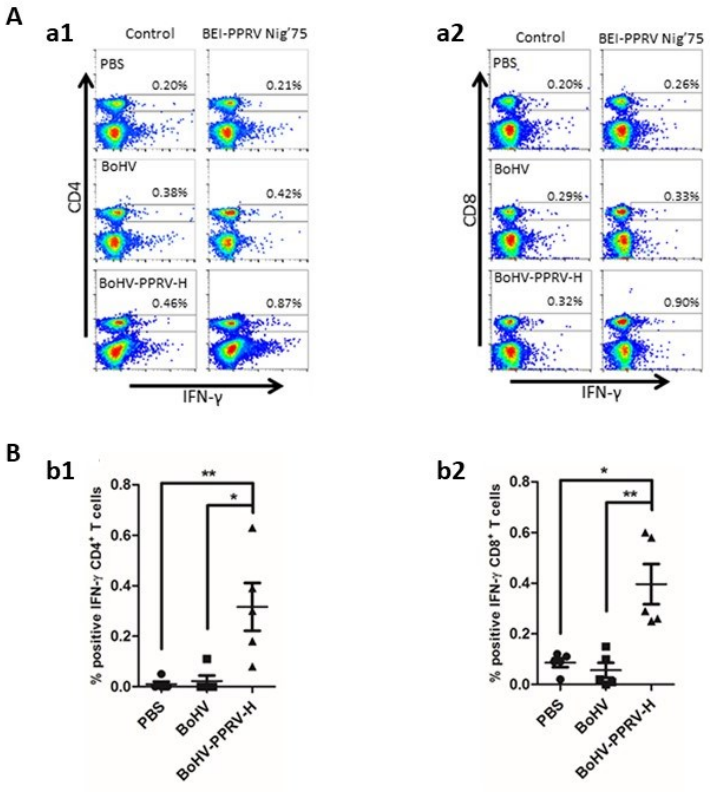

***Figure 31*. Anti-PPRV T Cell response induced by BoHV-4-A-PPRV-H-ΔTK immunization.**

A. Flow citometry dot-plots of IFN-γ production from PBS-, BoHV-4-A- ("BoHV" in the img.) and BoHV-4-A-PPRV-H-ΔTK- ("BoHV-PPRV-H" in the img.) immunized C57BL/6 mice CD4+ (**a1**)

and CD8+ (**a2**) T cells, in response to inactivated PPRV Nig'75 strain stimulation. Both CD4+ and CD8+ T cells from BoHV-4-A-PPRV-H-ΔTK vaccinated mice produced IFN-γ against PPRV, compared to PBS- and BoHV-4-A- (BoHV) immunized control groups. **B.** Average ± SD percentage of IFN-γ producing CD4+ (**b1**) and CD8+ T (**b2**) cells, derived at 7 days post booster vaccination from PBS-, BoHV-4-A- (BoHV) and BoHV-4-A-PPRV-H-ΔTK- (BoHV-PPRV-H) vaccinated C57BL/6 mice and stimulated with the inactivated PPRV Nig'75 strain. Data obtained using the Mann-Whitney test (*$p<0.05$; **$p<0.01$). All data have been measured above spontaneous IFN-γ control release in five mice per group.

Specific anti-PPRV-H epitopes T cell response induced by BoHV-4-A-PPRV-H-ΔTK immunization

In order to verify the T cell immune response specificity against the PPRV-H immuno-dominant antigen, splenocytes from PBS-, BoHV-4-A- and BoHV-4-A-PPRV-H-ΔTK-immunized C57BL/6 mice have been 1 week *in vitro* stimulated with PPRV-H H5 and H9 peptides (José Manuel Rojas et al. 2017), before IFN-γ production assession by flow cytometry. As a result, H5 peptide stimulation has induced specific IFN-γ production in CD8+ T cells, but not in CD4+ T cells, of BoHV-4-A-PPRV-H-ΔTK-immunized mice, while no specific H5 peptide-stimulated IFN-γ production has been detected in PBS- or BoHV-4-A-vaccinated mice splenocytes (*Fig.32*).

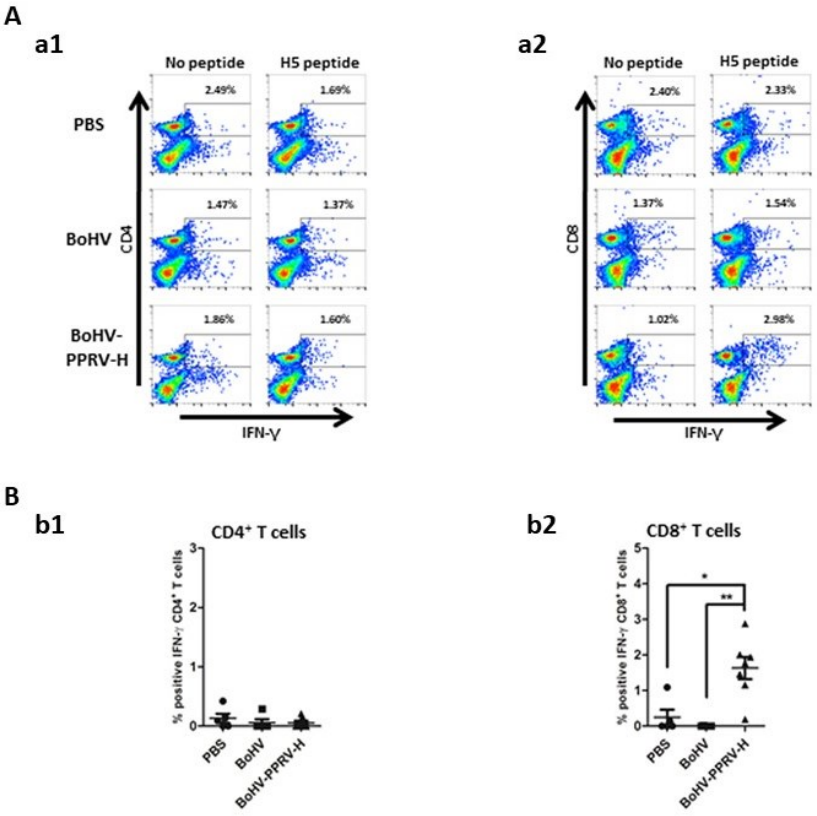

***Figure 32*. Specific anti-PPRV-H epitopes T cell response induced by BoHV-4-A-PPRV-H-ΔTK immunization.**

A. Flow citometry dot-plots of specific IFN-γ production from PBS-, BoHV-4-A- ("BoHV" in the img.) and BoHV-4-A-PPRV-H-ΔTK- ("BoHV-PPRV-H" in the img.) immunized C57BL/6 mice CD4+ (**a1**) and CD8+ T (**a2**) cells, in response to H5 peptide stimulation. Only CD8+ T cells from BoHV-4-A-PPRV-H-ΔTK vaccinated mice produced IFN-γ against H5, compared to PBS- and BoHV-4-A- (BoHV) immunized control groups. No IFN-γ production against H5 peptide has been detected from BoHV-4-A-PPRV-H-ΔTK vaccinated mice CD4+ T cells. **B.** Average ± SD percentage of specific IFN-γ producing CD4+ (**b1**) and CD8+ (**b2**) T cells against H5 peptide, from PBS-, BoHV-4-A- (BoHV) and BoHV-4-A-PPRV-H-ΔTK- (BoHV-PPRV-H) vaccinated C57BL/6 mice. Data obtained using the Mann-Whitney test (*$p<0.05$; **$p<0.01$). These data have been measured above spontaneous IFN-γ control release in five mice per PBS- and BoHV-4-A- (BoHV) groups and seven animals per BoHV-4-A-PPRV-H-ΔTK- (BoHV-PPRV-H) group.

However, H9 peptide stimulation has induced specific IFN-γ production in both BoHV-4-A-PPRV-H-ΔTK-immunized mice CD8+ T and CD4+ T cells, while no H9 peptide-stimulated IFN-γ secretion has been detected in PBS- or BoHV-4-A-groups. These data

demonstrate that BoHV-4-A-PPRV-H-ΔTK immunization elicit both specific CD8+ T and CD4+ T cells immune response against PPRV-H epitopes in C57BL/6 mice (*Fig.33*).

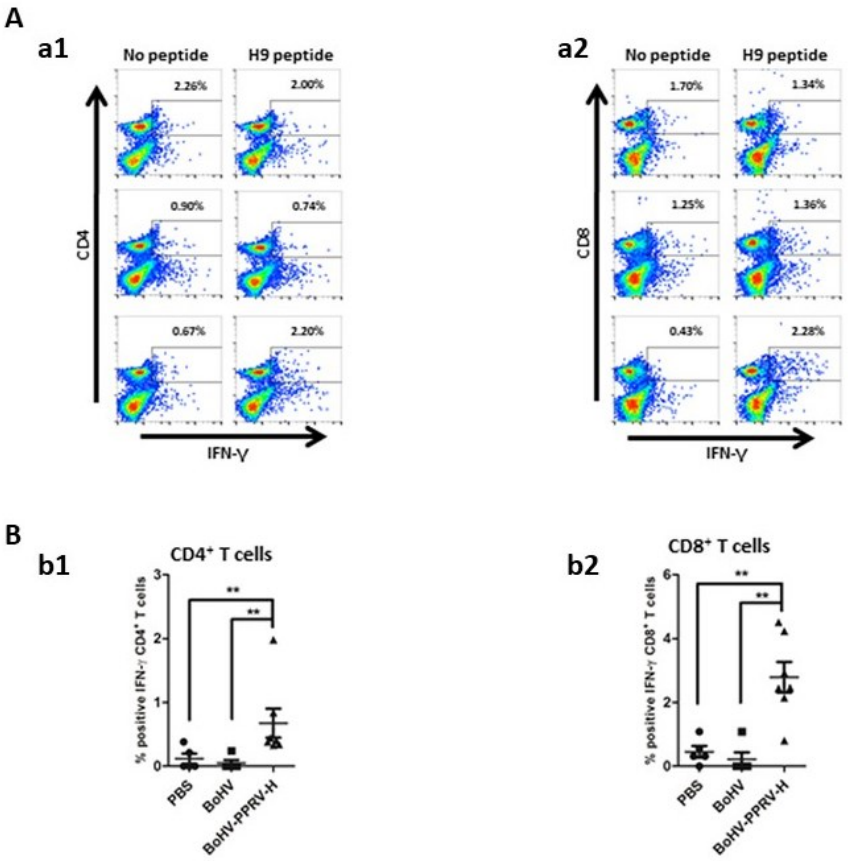

Figure 33. **Specific anti-PPRV-H epitopes T cell response induced by BoHV-4-A-PPRV-H-ΔTK immunization.**

A. Flow citometry dot-plots of specific IFN-γ production from PBS-, BoHV-4-A- ("BoHV" in the img.) and BoHV-4-A-PPRV-H-ΔTK- ("BoHV-PPRV-H" in the img.) immunized C57BL/6 mice CD4+ (**a1**) and CD8+ (**a2**) T cells, in response to H9 peptide stimulation. Only CD8+ T cells from BoHV-4-A-PPRV-H-ΔTK vaccinated mice produced IFN-γ against H9, compared to PBS- and BoHV-4-A- (BoHV) immunized control groups. No IFN-γ production against H9 peptide has been detected from BoHV-4-A-PPRV-H-ΔTK vaccinated mice CD4+ T cells. **B.** Average ± SD percentage of specific IFN-γ producing CD4+ (**b1**) and CD8+ (**b2**) T cells against H9 peptide, from PBS-, BoHV-4-A- (BoHV) and BoHV-4-A-PPRV-H-ΔTK- (BoHV-PPRV-H) vaccinated C57BL/6 mice. Data obtained using the Mann-Whitney test (*$p<0.05$; **$p<0.01$). These data have been measured above spontaneous IFN-γ control release in five mice per PBS- and BoHV-4-A- (BoHV) groups and seven animals per BoHV-4-A-PPRV-H-ΔTK- (BoHV-PPRV-H) group.

Cytotoxic T lymphocytes (CTL) response induced by BoHV-4-A-PPRV-H-ΔTK immunization

BoHV-4-A-PPRV-H-ΔTK vaccination induced Cytotoxic T lymphocytes immune response has been evaluated by flow cytometry-based cytotoxicity assay. BoHV-4-A-PPRV-H-ΔTK-immunized mice splenocytes have been previously H5 peptide *in vitro* stimulated for one week and exploited as the assay effector cells, while RMA-s cells have been used as target cells. RMA-s cell membrane has been fluorescently labeled with the PKH67 marker, and cells have been H5 peptide pulsed or left unpulsed as control. Pulsed and unpulsed labeled RMA-s cells have then been incubated with the splenocyte effector cells. Target cells lysis have been evaluated by Propidium Iodide (PI) staining. As a result, BoHV-4-A-PPRV-H-ΔTK-immunized mice splenocytes specifically lysed the H5 peptide pulsed RMA-s cells, indicating that BoHV-4-A-PPRV-H-ΔTK immunization in C57BL/6 mice promotes specific CTL responses against PPRV infection (*Fig.34,35*).

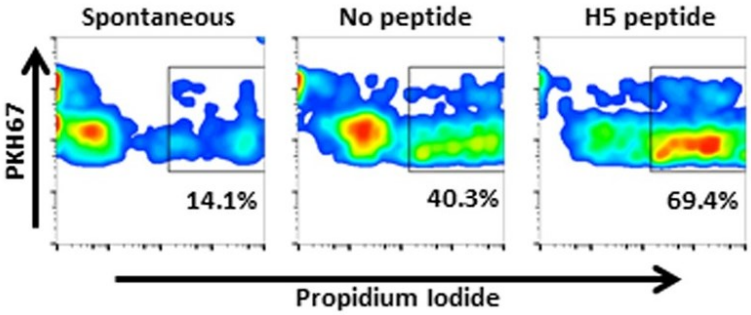

Figure 34. **Specific CTL responses evaluation by flow cytometry-based cytotoxicity assay of PKH67 marked RMA-s cells lysis after incubation with H5 peptide pulsed and unpulsed BoHV-4-A-PPRV-H-ΔTK-immunized mice splenocytes.**

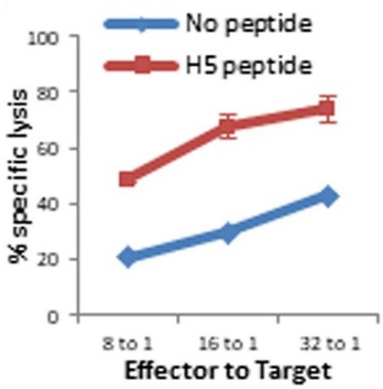

***Figure 35*. Specific % cytotoxicity of H5 peptide stimulated BoHV-4-A-PPRV-H-ΔTK-immunized mice splenocytes cultured at various ratio with H5 peptide pulsed target cells.**

Specific anti-PPRV-H neutralizing antibody response induced by BoHV-4-A-PPRV-H-ΔTK immunization

BoHV-4-A-PPRV-H-ΔTK-induced humoral immune response in C57BL/6 mice has been evaluated by virus neutralization assay. Sera from BoHV-4-A-PPRV-H-ΔTK vaccinated mice have been collected at 7 days after boost immunization (28 total days post first inoculation) to determine the presence of anti-PPRV.H neutralizing antibodies. As a result, all ten BoHV-4-A-PPRV-H-ΔTK immunized mice produced high titers, between 160 and 320, of specific anti-PPRV-H neutralizing antibodies. Pre-immune and PBS or BoHV-4-A-injected mice sera served as negative controls and did not show any neutralization activity (*Fig.36*). These data demonstrate that C57BL/6 mice *in vivo* inoculation of recombinant BoHV-4-A expressing the PPRV-H antigen stimulates the production of PPRV neutralizing antibodies, suggesting this vaccination approach as potentially capable to confer protective immunity toimmunized animals.

In vitro analysis of neutralization of Nigeria 75/1 peste des petits ruminants virus (PPRV) strain infectivity.

Neutralization titer[a]			
Source of antigen[b]	**Mouse no.**	**0 DPI[c]**	**28 DPI[d]**
PBS	1 to 10	<10	<10
BoHV-4A	1 to 10	<10	<10
BoHV-4-PPRV-H	1	<10	120
	2	<10	360
	3	<10	120
	4	<10	360
	5	<10	120
	6	<10	360
	7	<10	120
	8	<10	120
	9	<10	120
	10	<10	120

[a] *SN titers were determined against Nigeria 75/1 PPRV strain and expressed as the reciprocal of the last dilution of serum that neutralized 50% of the virus-specific cytopathic effect in flat bottom 96 plates.*

[b] *Intraperitoneally inoculations of different antigens (see Materials and Methods).*

[c] *Analyzed sera obtained from uninfected mice.*

[d] *Analyzed sera obtained from infected mice 28 days post first immunization (7 days post-boost).*

***Figure 36. In vitro* analysis of Nigeria 75/1 PPRV strain infectivity neutralization.**

DISCUSSION AND CONCLUSION

Peste des Petit Ruminants (PPR) disease is an endemic infectious disease that has been first reported in West Africa in the 1940s. Since then it has been spreading across North and East Africa, and to the Near and Middle East, reaching the South and East Asia, where it has been reported in China in 2007, while recently, in 2016, it has also emerged in Europe.

PPR is caused by a negative single stranded RNA-based virus, globally known as Peste des Petit Ruminants Virus (PPRV). PPRV belongs to the *Morbillivirus* genus, *Paramyxoviridae* family and it affects both wild and domestic small ruminants, primarily goats and sheep, causing fever, oral lesions, diarrhea, pneumonia and, often, death.

Indeed, PPR represents a highly concerning disease for developing countries populations, mostly engaged in small-scale farming, where almost the 80% is constituted by sheep and goats livestock. To date, PPR constitutes an Office International des Epizooties (OIE)-listed disease (http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2018/). Investing in PPR eradication would significantly meet the Sustainable Development Goals (SDGs) objectives, contributing to food security, by increasing milk and meat production, and reducing vulnerable pastoral and rural communities poverty, by increasing household benefits and healthcare. Indeed, the OIE and the Food and Agriculture Organization of the United Nations (FAO) have jointly developed a Global Strategy for PPR control and eradication, expected to be reached by 2030. Vaccination represents the most effective strategy for reducing animal mortality, increasing livestock productivity and reviving undeveloped areas populations economy (Marsh et al. 2016). Particularly, an effective vaccine targeting diseases that hamper developing countries farming, needs to combine vaccination with sero-surveillance. Gene deleted marker- (Ahrens et al. 2000), sub-unit- (de Smit et al. 2001), heterologous- (Capua et al. 2003), and recombinant vector based-vaccines, all represent Differentiating Infected from Vaccinated Animals (DIVA) vaccinations, capable of overcoming classical live attenuated vaccines inability to distinguish between vaccination- and natural infection-stimulated immunities.

The objective of this PhD project is a DIVA viral vectored vaccine that potentially meets the Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (OIE) global criteria for PPR sero-surveillance, control and eradication. Specifically, a Bovine Herpesvirus type 4 (BoHV-4) -based vector platform has been recombined via heat inducible homologous BAC recombineering system for *in vivo* PPRV gene delivery and expression in immunocompetent mice. Although there are no PPRV

disease-representative murine models, C57BL/6 mice have been choosen as surrogate animal models to quickly and cheaply obtain preliminary data wether this recombinant BoHV-4 based vaccine is capable of *in vivo* inducing immunity against the PPR disease. The next experimentation step will be to verify the predictivity of the BoHV-4-based vaccine efficacy on PPRV natural host, whose experimentation require greater efforts in terms of costs, timing and biosafety containment structures (José M. Rojas, Moreno, et al. 2014; José Manuel Rojas et al. 2017). The PPRV Hemagglutinin (PPRV-H) glycoprotein represents the virus surface immunodominant antigen, possessing both hemagglutinin and neuraminidase activities, that has already been widely exployited as a good vaccine candidate antigen (Herbert et al. 2014; José Manuel Rojas et al. 2017). The mature H protein possesses an amino acids 35-38 N-terminal hydrophobic domain acting as a signal peptide that anchors the protein into the membrane and 34 N-terminal transmembrane amino acids that characterize PPRV-H as a type II glycoprotein (Langedijk, Daus, e van Oirschot 1997). Based on these data, a recombinant PPRV-H BoHV-4-based vector (BoHV-4-A-PPRV-H-ΔTK) vaccine, delivering an optimized PPRV-H expression cassette, has been *in vitro* generated and *in vivo* tested as a potential DIVA vaccine platform for PPR vaccination.

BoHV-4 presents several interesting characteristics that suggest it as a potential gene therapy delivery vector. Several experimental works based on recombinant BoHV-4s employied for vaccination and oncolytic purposes prove its potential effectiveness as gene delivery vector. However, it has often been associated, together with specific endometotropism, to cattle postpartum metritis (Frazier et al. 2002; Monge et al. 2006), but since there are no direct proof of BoHV-4 pathogenicity, its involvement in uterine diseases has been explained with an indirect pathogenic model, where BoHV-4-caused damages are induced by the release of Prostaglandin E2 (PGE2), Tumor Necrosis Factor α (TNF-α), and Lipopolysaccharide (LPS) from a previous bacterial infection (Gaetano Donofrio et al. 2008; Jacca et al. 2013). The BoHV-4-A strain employed in this thesis project has been isolated from the milk cell fraction of a healthy cow and its genome has been first cloned as a Bacterial Artificial Chromosome (pBAC-BoHV-4-A) and then recombined with the PPRV-H Open Reading Frame (ORF) placed under the CMV promoter transcriptional control, using BoHV-4-A Thymidine Kinase (TK) locus as homology regions (G. Donofrio et al. 2008). Moreover, BoHV-4-A-based vector has been proved to behave as a non-competent replicating viral vector in both Wild Type (WT) and immunocompromised mice, with complete absence of pathogenicity (Franceschi et al. 2011; Redaelli et al. 2012; Franceschi et al. 2015; Gaetano Donofrio, Cavaggioni, et al. 2006; Franceschi et al. 2014; Puppo et al. 2014).

The so generated, BoHV-4-A-PPRV-H-ΔTK, has been able of *in vitro* transducing mammalian cells and it expressed the PPRV-H protein, thus being potentially capable of eliciting immunity against the delivered transgene. The virus has also been proved to remain genetically stable over several culture passages. However, current laws do not legalize yet the employment of Genetically Modified Organisms (GMO) for field trials. Despite the current legal restrictions, this pilot C57BL/6 mice immunization with the DIVA BoHV-4-A-PPRV-H-ΔTK-based vaccine here experimented, has proved to stimulate a protective immunity against PPR disease, by inducing both humoral and adaptive immunity components.

BoHV-4-A-PPRV-H-ΔTK immunized mice efficiently produced specific helper CD4+ and cytotoxic CD8+ T cell responses against PPRV-H epitopes, promoted CTL responses against PPRV, and induced high titer of sero-neutralizing antibodies against PPRV-H. Helper $CD4^+$ T cells play a crucial immune role in presenting viral epitopes to plasma B cells while $CD8^+$ T cells cytotoxic activity assures virus clearance by directly eliminating the infected cells, limiting the virus dissemination. Cell mediated immune activity intertwined with the production of high titers of sero-neutralizing antibodies ensures a protective barrier against PPRV. Moreover, the stimulation of humoral immunity might also induce protection against the morbillivirus prototype Measles Virus (MV) re-infection (Mongkolsapaya et al. 1999; de Vries et al. 2010). The most striking result resulting from BoHV-4-A-PPRV-H-ΔTK vaccination, is the anti-PPRV Virus Neutralizing Antibodies (VNAs) production. Neutralization titer higher than 10 has been previously showed to define long lasting humoral protection, thus, titers over 10 represent a protection indicator for successfull field vaccination (Lund et al. 2000; Gans et al. 2013). The lowest VNA titers obtained for all ten BoHV-4-A-PPRV-H-ΔTK vaccinated mice were of 120, a titer much higher then 10, thus suggesting a potentially strong protection from virulent PPRV strain challenge also in PPRV natural hosts. Previously published experimental data about BoHV-4-based vaccines capability of inducing immunity in goats and sheep (Gaetano Donofrio, Sartori, et al. 2007; Gaetano Donofrio et al. 2013), increase BoHV-4-A-PPRV-H-ΔTK vaccination potentiality of stimulating long lasting immune protection in small ruminants against the PPR disease. Moreover, these BoHV-4-A-PPRV-H-ΔTK induced VNA titers are higher than the ones obtained from other viral vector-based vaccination delivering both PPRV-H and -Fusion peptide (F) (A. Diallo et al. 2002; Berhe et al. 2003). This data support the concept of paramyxoviruses H glycoprotein being a VNAs stronger inducer compared to F protein. Despite humoral immune response is considered the main factor for an efficient anti-PPRV

protection, there have been cases of protection in absence of detectable neutralizing antibodies. Therefore, in this specific case, the stimulation of both immunities, antibody- and cell-mediated, represents a very promising data (Jones et al. 1993; Saravanan et al. 2010). The induction of high VNA titer levels and the stimulation of T cell-mediated viral clearance after BoHV-4-A-PPRV-H-ΔTK immunization, suggests that this vaccine could potentially protect PPRV natural hosts from virulent challenge both on experimental setting and field.

An interesting future prospective will be to determine whether BoHV-4-A-PPRV-H-ΔTK immunization can trigger memory T cell responses in PPRV natural hosts, as well as recombinant adenovirus-based vaccines (José Manuel Rojas et al. 2017). Since we have obtained very promising preliminary data in mice, where BoHV-4-based vector behaves as a replication deficient vector, an immune response from BoHV-4-A-PPRV-H-ΔTK immunized sheep, where the virus is replication competent, would be expected to be increased. Immunization trials on sheep with ovine cell-associated BoHV-4-A-PPRV-H-ΔTK are currently underway at the "Centro de Investigacion en Sanidad Animal" (CISA-INIA), in Madrid.

Concluding, this PhD project data proved that the recombinant BoHV-4-A-PPRV-H-ΔTK so generated is capable of stimulating a strong and specific immunity, complete of innate and adaptive responses, in mice, against PPRV. These findings suggest recombinant BoHV-4-A-PPRV-H-ΔTK potential use for vaccination purposes on PPRV natural hosts, and further confirm BoHV-4 as a safe, large, potent and non-integrative viral vector, potentially employiable for PPR vaccination and eradication.

List of papers published during the PhD in Medical-Veterinary Science

BoHV-4-based vector delivering Ebola virus surface glycoprotein.
Rosamilia A, Jacca S, Tebaldi G, Tiberti S, Franceschi V, **Macchi F,** Cavirani S, Kobinger G, Knowles DP, Donofrio G.
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Bovine Herpesvirus-4-Based Vector Delivering Peste des Petits Ruminants Virus Hemagglutinin ORF Induces both Neutralizing Antibodies and Cytotoxic T Cell Responses.
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Bovine herpesvirus 4-based vector delivering the full length xCT DNA efficiently protects mice from mammary cancer metastases by targeting cancer stem cells
Gaetano Donofrio, Giulia Tebaldi, Stefania Lanzardo, Roberto Ruiu, Elisabetta Bolli, Andrea Ballatore, Valeria Rolih, **Francesca Macchi**, Laura Conti & Federica Cavallo
OncoImmunology Received 22 Feb 2018, Accepted 24 Jun 2018, Published online: 06 Sep 2018

Capacity to Elicit Cytotoxic CD8 T Cell Activity Against Mycobacterium avium subsp. Paratuberculosis Is Retained in a Vaccine Candidate 35 kDa Peptide Modified for Expression in Mammalian Cells
Valentina Franceschi, Asmaa H Mahmoud, Gaber S Abdellrazeq, Giulia Tebaldi, **Francesca Macchi**, Luca Russo, Lindsay M Fry, Mahmoud M Elnaggar, John P Bannantine, Kun-Taek Park, Victoria Hulubei, Sandro Cavirani, William C Davis, Gaetano Donofrio
Front Immunol. 2019; 10: 2859. Published online 2019 Dec 11. Doi:10.3389/fimmu.2019.02859.
PMCID: PMC6917596. PMID: 31921129

Immunotargeting of the xCT Cystine/Glutamate Antiporter Potentiates the Efficacy of HER2-Targeted Immunotherapies in Breast Cancer

Laura Conti, Elisabetta Bolli, Antonino Di Lorenzo, Valentina Franceschi, **Francesca Macchi,** Federica Riccardo, Roberto Ruiu, Luca Russo, Elena Quaglino, Gaetano Donofrio, Federica Cavallo

Cancer Immunol Res. 2020 Aug; 8(8):1039-1053. DOI: 10.1158/2326-6066.CIR-20-0082

Bovine Herpesvirus-4-Vectored Delivery of Nipah Virus Glycoproteins Enhances T Cell Immunogenicity in Pigs

Miriam Pedrera, **Francesca Macchi,** Rebecca K McLean, Valentina Franceschi, Nazia Thakur, Luca Russo, Lobna Medfai, Shawn Todd, Elma Z Tchilian, Jean-Christophe Audonnet, Keith Chappell, Ariel Isaacs, Daniel Watterson, Paul R Young, Glenn A Marsh, Dalan Bailey, Simon P Graham, Gaetano Donofrio

Vaccines (Basel) 2020 Mar 2;8(1):115. Doi: 10.3390/vaccines8010115

Conferences

VI NATIONAL VETERINARY VIROLOGY WORKSHOP

CAVALLERIZZA REALE, Turin, 13-14 October 2016

Oral presentation: "Bovine herpesvirus 4-based vector delivering a hybrid rat/human HER-2 oncoantigen 1 efficiently protects mice from autochthonous Her-2+ mammary cancer."

LXXI SISVET CONGRESS IN VETERINARY MEDICINE

Naples, from 28 June -1 July 2017

Oral presentation: "Enhanced oncolytic activity of BoHV-4-based vector delivering a miRNA sequence against enolase-2 in Glioblastoma Multiforme"

THE ROSLIN INSTITUTE, INTERNAL VIROLOGY SEMINAR

The Roslin Institute, Royal (Dick) Veterinary Studies, University of Edinburgh, Scotland, 6 June 2018

Oral presentation: "Roslin International Virology Seminar: Bovine Herpesvirus-4-Based Vector Delivering Peste des Petits Ruminants Virus Hemagglutinin ORF Induces both Neutralizing Antibodies and Cytotoxic T Cell Responses."

2nd NATIONAL CONGRESS OF THE ITALIAN SOCIETY FOR VIROLOGY

Rome,28-30 November 2018

Poster presentation: "Bovine Herpesvirus-4-Based Vector Delivering Peste des Petits Ruminants Virus Hemagglutinin ORF Induces both Neutralizing Antibodies and Cytotoxic T Cell Responses"

European Researchers' Night

The event aims at creating opportunities to bring together researchers and citizens in order to disseminate scientific culture and information about research careers in an informal and stimulating context

30 September 2016

28 September 2018

27 September 2019

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