

UNIVERSITA DEGLI STUDI DI PARMA

FACOLTA DI MEDICINA VETERINARIA
SEZIONE DI MALATTIE INFETTIVE

DOTTORATO DI RICERCA IN SALUTE ANIMALE
XXIII CICLO

Generation of molecular tools for Bluetongue virus (BTV)
diagnosis and immunization

Coordinatore:
Chiar.mo Prof. Sandro Cavirani

Tutor:
Chiar.mo Prof. Gaetano Donofrio

Dottorando:
Capocefalo Antonio

INDEX

INDEX.....	1
ABSTRACT.....	4
1. INTRODUCTION.....	6
1.1 Epidemiology.....	8
1.2 BTV Virus.....	14
1.2.1 BTV Structure.....	14
1.2.2 BTV Cycle Replicative.....	19
1.3 BTV Disease.....	27
1.3.1 Pathogenesis.....	27
1.3.2 Clinical Signs.....	30
1.3.3 Lesions.....	32
1.4 BTV Proteins Expression System.....	34
2. AIM OF WORK.....	39
3. MATERIALS AND METHODS.....	40
3.1 Polymerase Chain Reaction.....	40
3.2 Agarose gel electrophoresis.....	41
3.3 DNA extraction from an agarose gel.....	42
<i>Protocol DNA extraction gel:</i>	42
3.4 Cell cultures.....	43
<i>Cell culture passage protocol:</i>	44
3.5 Electroporation.....	44
<i>Generation of electrocompetent bacteria (DH10B or HS996):</i>	45
<i>Electrocompetent bacteria transformation protocol:</i>	45
<i>Animal cells electroporation protocol:</i>	46
3.6 Heat shock.....	47
<i>Hanahan Transformation of XL1Blue:</i>	47
<i>Heat Shock Protocol:</i>	47
3.7 Plasmid DNA purification.....	48
<i>Miniprep protocol:</i>	48
<i>Maxiprep protocol:</i>	49
3.8 Protein Extraction.....	50
<i>Protein Extraction protocol (for a 75 cm2 flask):</i>	51
3.9 Western blotting.....	52
<i>Western Blotting Protocol:</i>	55
3.10 BAC recombineering.....	57
3.11 BAC DNA purification.....	59
<i>BAC extraction protocol (Warming et al., 2005):</i>	59
3.12 Southern blotting.....	60
<i>Probe labeling reaction:</i>	61
<i>Southern blotting protocol:</i>	62
4. RESULTS AND DISCUSSIONS.....	64
4.1 BTV-8 VP2 cDNA from pBRT7VP2.....	64
4.2 pIgkVP2 generation.....	66
4.3 VP2 Tag generation.....	68
<i>pIgK-VP2-gD361</i>	69

<i>plgK-VP2-gD200</i>	70
<i>plgK-VP2-gD181</i>	71
<i>plgK-VP2-gD127</i>	72
<i>plgK-VP2-gD106</i>	73
<i>plgK-VP2-gD93</i>	74
<i>plgK-E2-gD106</i>	75
4.4 VP2 fragmentation	77
<i>plgK-VP2⁽⁴⁻¹⁹⁵⁰⁾-gD106</i>	77
<i>plgK-VP2⁽⁹⁷⁵⁻²⁸⁸³⁾-gD106</i>	78
<i>plgK-VP2⁽¹⁶⁻⁷⁵⁰⁾-gD106</i>	80
<i>plgK-VP2⁽⁷⁵⁰⁻¹⁴²⁵⁾-gD106</i>	81
<i>plgK-VP2⁽¹³⁹⁵⁻²¹⁰⁰⁾-gD106</i>	82
<i>plgK-VP2⁽²⁰⁷⁰⁻²⁸⁸³⁾-gD106</i>	83
4.5 VP2chimeric peptide generation	85
<i>plgK-E2⁽⁴⁻²⁸⁸³⁾-gD106</i>	85
<i>plgK-E2⁽¹⁶⁻⁷⁵⁰⁾-gD106</i>	86
<i>plgK-E2⁽⁷⁵⁰⁻¹⁴²⁵⁾-gD106</i>	87
<i>plgK-E2⁽¹³⁹⁵⁻²¹⁰⁰⁾-gD106</i>	88
<i>plgK-E2⁽²⁰⁷⁰⁻²⁸⁸³⁾-gD106</i>	88
4.6 BoHV-4 U ΔORF50 IgKE ₂ VP ₂ ²⁰⁷⁰⁻²⁸⁸³ gD ¹⁰⁶	90
5 CONCLUSIONS	95
BIBLIOGRAPHY	97

ABSTRACT

Bluetongue virus (BTV), the prototype member of the genus *Orbivirus* in the family *Reoviridae*, is an insect-borne virus that infects domestic and wild ruminants, causing a non contagious infectious disease spread by biting midges of some species of *Culicoides* genus, known as Bluetongue (BT).

Historically, BT occurs most commonly and seriously in sheep and in some species of wild ruminants, occasionally in goats and rarely in cattle. In 2006, BTV serotype 8 (BTV-8) emerged in Northern Europe followed by a still ongoing epidemic; the BTV-8 occurrence is remarkable for several reasons and, particularly, for its virulence not only in sheep between domestic ruminants.

Currently, BTV, and particularly BTV-8, is responsible of great farming industries losses; for this reason it is critical to have effective molecular tools to define standardized, specific and sensitive serodiagnostic assay and successful and economic prophylactic immunization plans.

BTV is a double-stranded RNA (dsRNA) virus and its genome consists of 10 dsRNA segments that encode 4 nonstructural proteins (NS1-NS3 and NS3A) and 7 structural proteins (VP1-VP7). Among the 7 structural proteins, VP2, the outermost viral protein, is the cellular binding protein, elicits virus-neutralizing antibody, and is responsible for hemagglutinin activity and serotype specificity.

Although VP2 has been expressed successfully through many systems and particularly with insect cell-baculovirus expression system, its paracrine expression as a soluble form in mammalian cells represents a difficult task.

In this work we have investigated a mammalian expression platform for BTV VP2 production as a soluble form, generating several expression vector plasmids with BTV-8 VP2 sequence, whole or fragmented, by fusion peptides strategy.

Starting from the assumption that VP2 is strongly cell associated, the full length of VP2 ORF was sub-cloned in frame with the immunoglobulin kappa light chain (IgK) signal peptide specifying secretion of heterologous proteins, generating plgkVP2. In order to attempt the expression of BTV-8 VP2 with plgkVP2 vector in mammalian cells and due to the lack of a suitable monoclonal antibody against BTV-8 VP2, a tag soluble epitope peptide belonging to the Bovine herpesvirus 1 (BoHV-1) glycoprotein D (gD) ectodomain was generated, obtaining plgkVP2gD106. After electroporation of HEK 293T cells, we found that VP2 was well expressed from the transfected cells, but not secreted into the medium,

showing that introduction of a heterologous signal peptide in the amino-terminus of VP2 is not sufficient to specify its secretion.

In previous studies was demonstrated that BTV VP2 associates with Vimentin cytoskeleton protein, and deletions of amino acids between residues 65 and 114 into VP2 sequence have shown to disrupt VP2-vimentin association. This aspect could be the cause of the missing VP2 secretion. Thus, new plasmid vectors was generated with different fragments of VP2 in frame upstream with Igk signal peptide, and downstream with gD106 tag; although all VP2 fragments are found in cell fraction, none is secreted into the medium.

Based on the fact that BVDV gE2 ectodomain was very well expressed and secreted by plgK-E2, gE2 ectodomain was exploited as a leader sequence to get VP2 and derived fragments secreted into the medium of transfected cells. Therefore, VP2 and derived fragments were sandwiched between IgkE2 and gD106 tag. We found that two constructs, plgkE₂VP₂⁷²⁰⁻¹⁴²⁵gD¹⁰⁶ and plgkE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶, successfully expressed and secreted the VP2 fragments.

Finally, BoHV-4 U ΔORF50 strain cloned as a bacterial artificial chromosome (BAC) was engineered to express VP₂²⁰⁷⁰⁻²⁸⁸³. Thus, we inserted the IgkE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ expression cassette into IE2 gene of a mutant BoHV-4 U ΔORF50 strain, in which IE2 locus is duplicated and one of them is inactivated by the insertion of 2004 bp KanaGalk DNA sequence stuffer double selecting cassette. So we obtained a recombinant BoHV-4 strain virus-vector able to yeald hight quantity of soluble VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ fragment from infected cells, which could be employed for immunodiagnostic assay development or vaccine purposes.

1. INTRODUCTION

Bluetongue Virus (BTV) is the aetiological agent of Bluetongue (BT) disease, a non-contagious, arthropod-borne, viral disease of domestic and wild ruminants^{1,2}.

BT disease occurs most commonly and seriously in sheep and in some species of wild ruminants, such as white-tailed deer (*Odocoileus virginianus*), pronghorn (*Antilocapra americana*) and desert bighorn sheep (*Ovis canadensis*)³, in which BTV infection causes an acute disease with high morbidity and mortality. In these species the disease reflects virus-mediated microvascular injury, causing congestion, cyanosis, ulceration, haemorrhage and tissue infarction. The disease is characterized by catarrhal inflammation of digestive and respiratory apparatus; necrosis of skeletal striated muscular tissue and necrosis of cardiac striated muscular tissue; laminitis and coronitis; finally, abortion, mummified fetal and fetal abnormalities may occur after infection of ewes during early pregnancy⁴.

In contrast, clinical disease is seen occasionally in goats and rarely in cattle: BTV infection of the cattle and goats is typically asymptomatic and, since viremia can persist for several weeks, these animals are considered a major reservoir of BTV. Sporadic occurrence of disease in BTV-infected cattle has been attributed to an IgE-mediated hypersensitivity reaction⁴.

BTV is the type species of the genus Orbivirus, a member of a Reoviridae family. The Reoviridae (Reo: sigla from Respiratory Enteric Orphan, due to the early recognition that the viruses caused respiratory and enteric infectious and the, incorrect, belief that they were not associated with specific disease, hence they were considered *orphan* viruses, Sabin 1959) family is one of the largest families of viruses and includes a total of 12 distinct genera. Many of these viruses are pathogenic to animals and some also infect or are pathogenic for humans (e.g. Orthoreovirus, Rotavirus, Coltivirus and Seadornavirus) as well as for plants and insects⁵.

The members of the Reoviridae family are non-enveloped particles with icosahedral symmetry, but may appear spherical in shape. The protein capsid is organized as one, two, or three concentric capsid layers, which surround the dsRNA segments of the viral genome, with an overall diameter of 60-80 nm. Virions contain 10,11, or 12 segments of linear dsRNA, depending on the genus. The twelve genera can be divided between two groups. One group contains viruses which have relatively large “spikes” or “turrets”

situated at the 12 icosahedral vertices of either the virus or core particle, including: Orthoreovirus, Cypovirus, Aquareovirus, Fijivirus, Oryzavirus, Idnoreovirus and Mycoreovirus, as well as some of the unclassified or unassigned viruses from invertebrates. The second group includes those genera containing smooth viruses that do not have these large surface projections on their virions or core particles, giving them an almost spherical appearance, including Orbiviruses, Rotavirus, Coltivirus, Seadornavirus and Phytoreovirus⁵.

The Orbiviruses (Orbi: from latin orbis, “ring” or “circle” in recognition of the ring-like structures observed in micrographs of the surface of core particles), which encompass, besides BTV, the agent causing African horse sickness (AHSV), equine encephalosis virus (EEV) and epizootic hemorrhagic disease of deer (EHDV), have a 10 segmented double-stranded RNA (dsRNA) genome contained within a three-layered icosahedral protein capsid; these viruses infect animals, plants and insects and are all transmitted by arthropods, such as mosquitoes, gnats and ticks. Many of these viruses cause diseases in animals or plants, often with high economic impacts in agriculture and animal health⁵.

BTV is the prototype of the Orbivirus genus and it encompasses currently at least 24 serotypes, with a probable 25th recently identified amongst goats in Switzerland^{6,7}. The vectors are species of hematophagous midges of the genus *Culicoides* and the geographical distribution and the temporal incidence of the different serotypes and strains of BTV are therefore linked to the occurrence of distinct species of competent *Culicoides* insect vectors^{3,8}.

BTV has been isolated from ruminants and/or vector insects from all continents except Antarctica, but, although BTV likely has long been present throughout tropical, subtropical and some temperate regions of the world, the global distribution of BTV infection very recently has drastically changed by spreading to previously unaffected parts of the world such as most of Europe^{8,9}. It has been proposed that climate change is in part responsible for this profound change in the global BTV diffusion, presumably by its impact on the vectorial capacity of resident *Culicoides* insect populations in previously virus-free regions^{3,10}. BTV has recently spread throughout most of Europe where, until approximately 1998, only sporadic and transient epizootics of BTV infection occurred previously and in 2006 BTV serotype 8 emerged in northern Europe followed by a still ongoing epidemic. The BTV-8 occurrence is remarkable for its virulence, not only in sheep between domestic ruminants, and for its broad spread.

For its wide and growing spread and its economic impact, BTV has been placed on list-A by the Office International des Epizooties (OIE)¹¹.

1.1 Epidemiology

Historically, Bluetongue virus was thought to be confined to southern Africa, where it has probably been endemic in wild ruminants from antiquity. Bluetongue, as a disease in cattle and sheep, was first described as “Fever”, “Malarial Catarrhal Fever” or “Epizootic Catarrh of Sheep” in the original descriptions of investigators in South Africa^{1,12,13}. Although the original written descriptions of BT were published in the late 19th and early 20th centuries, the disease was likely recognized as soon as wool European sheep breeds, that are highly susceptible to the disease and particularly Merino sheep, were introduced to southern Africa^{1,3,13}. A disease whose clinical features resemble the BT was described, as “Tong-Sikte”, by a French biologist Francois de Vaillant during his travels in the Cape of Good Hope between 1781 and 1784¹³. Henning notes that the Report of the Cattle and Sheep Disease for 1876¹⁴ states: *“For many years, if not from the time of the introduction of the Merino sheep into the colony, there has been prevalent amongst the flocks a disease known as Fever”*^{1,15}. But the first comprehensive clinical profile of the disease was published by Duncan Hutcheon, the Chief Veterinary Officer of the Cape Colony; he recorded some clinical features of the disease in his annual report for 1880, referring to it as Fever or Epizootic Catarrh in sheep^{1,13,16}. In 1902, he wrote of Malarial Catarrhal Fever in sheep, in the Veterinary Record, where he postulated that the disease agent was insect-transmitted^{13,17}. Then in 1905, Spreull was the first to study the disease in detail; he described a typical case of the disease, the onset of which was marked by high fever and, after 7-10 days, distinctive lesions appeared in the mouth and the tongue turned dark blue, so he suggested that the name of the disease should be changed from Malarial Catarrhal Fever to Bluetongue^{1,13,18}. He showed also that the BT agent was transmissible to cattle and goats, but the infection in these ruminant species was apparently subclinical^{2,13,18}. Successively, Theiler first showed that the agent of BT was filterable and so its viral aetiology^{13,19}. The latter publication is still remarkable for the detailed and accurate description of the viral agent, clinical signs and pathology².

Prior to the 1940s, BTV was thought to be evolved and confined to southern Africa, but it was subsequently recognized in areas between latitude 40° N and 35° S in almost all

continents: America, Africa, Australia and Asia^{9,20}. More recently, there has been a dramatic northwards extension of the virus in Europe^{9,13,20}.

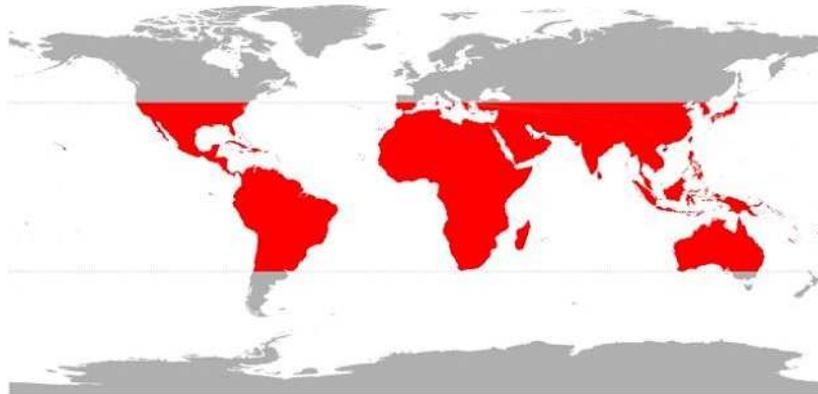


Figure 1.1 Map of the estimated global range of Bluetongue virus prior to its Europe spread. Figure from Wilson and Mellor, 2009⁹.

The first well-documented epizootic of BT outside of Africa occurred amongst sheep on Cyprus in 1943. However, it is believed that periodic unconfirmed outbreaks were occurring in Cyprus since 1924^{13,20,21}. The severity of the disease in Cyprus in 1943-1944, when around 2500 sheep died with some flocks experiencing 70% mortality, focussing international attention on this “new disease” coming out from Africa¹³. Further outbreaks of BTV, in Palestine in 1943, in Turkey in 1944, 1946 and 1947 and in Israel in 1951, served to reinforce the idea that this new disease was ‘emerging’^{9,13}. The following year, this concept was strengthened by the realization that ‘sore muzzle’, a new disease of sheep in California and Texas in North America which aetiology had not yet been established, was caused by BTV serotype 10, a type of BTV previously identified in South Africa^{9,13,23}. The detection of BTV in the USA represented an even more dramatic expansion in the known distribution of the virus.

Successively, BTV was first confirmed in Asia during 1961 when an outbreak of the disease was detected in India^{9,13,24}; then in 1977, it was isolated in Australia from *Culicoides* captured near Darwin two years earlier^{9,13,25}.

Although cases of BT have probably occurred regularly in Cyprus since at least 1924²¹, BTV otherwise caused only two outbreaks in Europe before 1998: the first and major occurrence of BTV-10 in Europe was in 1956, when the disease was diagnosed in southern Portugal and shortly thereafter also in Spain in 1957 and continued in the Iberian Peninsula until 1960. The outbreak was extremely severe with over 180000 sheep dying and with a mortality rate around 75% being recorded in affected sheep. The outbreak was eventually controlled by imposing animal restriction, slaughter of infected individuals and

compulsory annual vaccination with a live vaccinated^{9,13,22}. A smaller outbreak of BTV-4 occurred in some Greek islands in the Aegean in 1978-1980²⁶. Despite the severity of the 1956-1960 outbreak, the virus remained an infrequent visitor to Europe for most of the twentieth century¹⁰. The situation changed dramatically from 1998, when BTV-9 was detected on several Greek islands close to the Turkish coast. Over the following 3 years, it spread northwards and westwards through mainland Greece and beyond²⁷, eventually reaching as far as Kosovo in 2001²⁸; this incursion was followed by incursions of BTV-1, BTV-4 and BTV-16^{9,10}. BTV-16 was isolated in Turkey in 2000 and occurs regularly in Israel, suggesting a Middle Eastern origin, while the strain of BTV-1 detected in 2001 is similar to isolates from India and Malaysia; this shows that the introduction of serotypes 1 and 19 were probably attributable at least in part to animal movements along the Eurasian movement street, a contiguous region with high densities of ruminants stretching from India and Pakistan through Afghanistan, Turkey, Iraq and Iran to the southeast of Europe. On the other hand, the BTV-4 strain identified in Greece in 1999 is closely related to strains recorded in the 1960 and in 1970 from Cyprus and Turkey, suggesting that the virus has been circulating in the region for many years^{10,20}. Meanwhile, BTV-2 entered Tunisia in 1999, spreading to Algeria and Morocco in 2000 and then onwards into the western and central Mediterranean islands and mainland Italy. A second strain of BTV-4, distinct from that in the eastern Mediterranean²⁹, also entered Menorca from North Africa in 2003. This same strain continued to cause outbreaks in Morocco in 2004 and spread to the Iberian Peninsula and Corsica in 2004 and 2005. In 2006, a western strain of BTV-1 was detected in North Africa³⁰ and subsequently spread to the Sardinia and Spain. There is therefore evidence that successful introductions of BTV into southern Europe via three routes have occurred nearly every year since 1998 and one or more serotypes has been active in southern Europe every year since 1998¹⁰.



Figure 1.2 The three principal routes by which Bluetongue virus is introduced in Europe. A, Morocco-Spain; B, Tunisia-Sicily; C, Turkey-Greece/Bulgaria. Figure from Wilson and Mellor, 2008¹⁰.

In August 2006, the Dutch Central Institute for Animal Disease Control (CIDC) in Lelystad reported the first ever case of BT in northern Europe. The virus responsible was subsequently identified at the UK Institute for Animal Health (IAH) as BTV-8, and subsequent sequence analysis suggested a close similarity to strains of this serotype previously isolated from sub-Saharan Africa³¹. During the following months, this outbreak spread to infect animals on over 2000 holdings, most in the Netherlands, Belgium, Germany, France and Luxembourg, before cold weather interrupted transmission by preventing the completion of viral incubation in the vector and reducing the activity of adult *Culicoides*³². The last clinical case was reported on 15 January 2007³³, although several further outbreaks were reported retrospectively as a result of serological testing^{9,10}. Because typical winter temperatures in Northern Europe are considerably lower than the minimum temperature required for BTV transmission, many hoped that the outbreak would be extinguished in the winter of 2006. Instead, the ability of BTV outbreaks to re-emerge after long periods of winter absence has been recognized for decades, and on 13 June 2007, the German National Reference Laboratory at the Friedrich Loeffler Institut confirmed that a sentinel animal on a holding in North-Rhine Westphalia had seroconverted between early April and early May³⁴, indicating that the virus had successfully overwintered in the region. The virus subsequently resurfaced in all countries affected in 2006, with new cases occurring for the first time in Denmark³⁵, Switzerland³⁶, the Czech Republic³⁷ and the UK³⁸. The spread of BTV-8 is remarkable for several reasons: (1) this virus has spread further north than BTV has ever previously been reported, (2) this virus is spread by Palearctic *Culicoides* species, and not *Culicoides imicola*, which is a major vector of BTV in Africa and Asia, (3) the strain of BTV-8 that invaded northern Europe is highly virulent, not only for sheep but also cattle³, and (4) it shows frequently vertical transmission in pregnant ruminants, a rare event in other BTV strains³⁹.

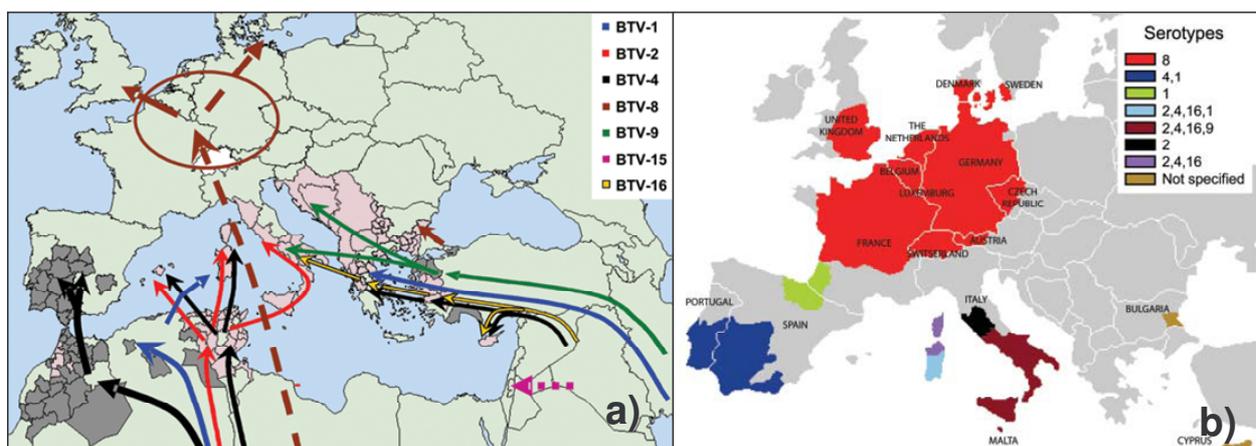


Figure 1.3 The epidemiology of different serotypes in Europe since 1998: in a) the routes of introduction of different serotype and in b) their spread⁴⁰.

The current outbreaks of BTV, and particularly BTV-8, are responsible of great farming industries losses in Europe: during 2008, for example, over 27000 holdings were affected by BTV-8 and over 6000 by BTV-1. In addition, two new serotypes arrived in Europe: BTV-11⁴¹ and in late 2008, bluetongue virus (BTV) serotype 6, which had never occurred in Europe before, was first detected in the Netherlands and Germany⁴².

Although venereal and vertical transmission of BTV can occur in ruminants⁴², with the notable exception of BTV-8 in Europe these events are apparently rare and considered unimportant to the maintenance of BTV; BT disease is not contagious and the infection is transmitted between its mammalian hosts by certain species of biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae). Infection occurs when adult females blood-feeds to obtain protein for the production of eggs. During a blood meal from an infected host, the virus passes into the lumen of the mid-gut of the midge; the virus must first infect cells of the posterior mid-gut wall and so progeny virus particles are then released into the insect's body cavity, from where they spread to the salivary glands, in which a second cycle of replication takes place. Virus is then available for injection along with saliva during subsequent blood-feeding on a new host¹⁰. *Culicoides* species are small (<3 mm) insects that occur in every inhabited continent in the world and breed in a wide variety of semi-aquatic sites; they differ between regions of the world and each specie is responsible to spread of different serotypes in different regions of the world. Of more than one thousand species of *Culicoides* that occur worldwide, only 30 or so have been incriminated as potential vector of BTV infection⁴³. The most important are *C. imicola* (the principal vector in Africa, Asia and most parts of Southern Europe), *C. sonorensis* (principal vector in North America) *C. insignis* (principal vector in Central and South America), *C. wadai* and *C. brevitarsis* (principal vector in Australia). Certainly other species, particularly in Africa, Asia and Australia play a role in BTV transmission in specific regions and at specific times, such as *C. fulvus* and *C. schultzei* in Asia and *C. bolitinos* in South Africa, among others⁹. Currently, new *Culicoides* species, named Palearctic species, have become important in BTV transmission in areas where BTV has ever previously been reported¹⁰. Vector population is influenced by several climatic conditions, such as temperature, humidity, rainfall and windy and different species of *Culicoides* have different environmental tolerance. So recently climate change is in part responsible for the drastic change in the global distribution of BTV. The rapid spread that followed the initial outbreaks of BTV-8 in Northern Europe in 2006 is generally considered a logical consequence of the changes in

global climate. These changes have allowed the Northern European midges, Palearctic species, such as *C. obsoletus*, an extremely small midge reaching only 0.8 mm in length and the most abundant of the Culicoides species in Northern Europe, and *C. pulicaris* to become effective transmitters of the virus.

All ruminants are probably susceptible to BTV infection including: cattle, sheep, goats, dromedaries and wild ruminants, but the disease occurs principally in sheep and some species of wild ruminants such as white-tailed deer (*Odocoileus virginianus*), pronghorn (*Antilocapra americana*) desert bighorn sheep (*Ovis canadensis*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), mouflon (*Ovis aries*), fallow deer (*Dama dama*), and Aoudad (*Ammotragus lervia*)⁴⁴⁻⁴⁷. Sheep that are native to tropical and subtropical regions of the world where BTV is endemic are usually resistant to BT disease, whereas fine wool European breeds such as the Merino are highly susceptible. BT disease is seen occasionally in goats and rarely in cattle, but generally the incidence of clinical disease varies by serotype, strain and host species and recently Europe BTV-8 occurrence is a major example.

1.2 BTV Virus

1.2.1 BTV Structure

BTV is a large (≈ 850 Å diameter) and architecturally complex virus. BTV virion is non-enveloped; it is spherical in appearance, but has icosahedral symmetry. There are seven structural protein^{5,48,49} (VP1-VP7) organized into two concentric layers, the outer and the inner capsid, around the viral genome^{5,50}: the outer capsid consists of two structural protein VP2 and VP5, while the inner capsid is composed of two major structural protein, VP3 and VP7, surrounding the three minor structural proteins, VP1, VP4 and VP6, and the viral genome; this is the viral core, a transcriptionally active compartment.

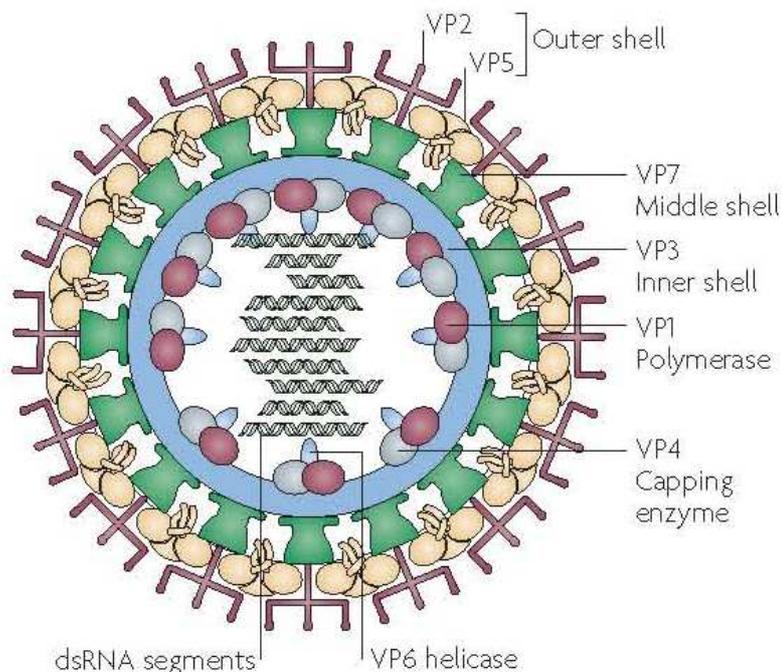


Figure 1.4 Bluetongue virus morphology. The bluetongue virus particle consists of the double-stranded RNA (dsRNA) genome surrounded by three concentric protein layers. The viral genome is divided into ten linear dsRNA molecules, each of which encodes a single protein and is located at the centre of the particle. The viral genome is surrounded by the icosahedral inner protein layer, which consists of VP3. On the inner surface of this layer, at the five-fold symmetry axes of the particle, are the RNA-dependent RNA polymerase (VP1) and capping enzyme (VP4). A viral helicase (VP6) is probably also associated with the VP1–VP4–RNA complex. The VP3 layer is surrounded by VP7, a trimeric protein. Contacts between these two layers are formed by non-specific hydrophobic interactions. The outer layer (which consists of VP2 and VP5) of the virus particle is responsible for attachment⁵¹.

VP2 and VP5, the outer capsid proteins, are responsible for virus entry and penetration in host cell⁵². In fact, outer layer is removed⁵² during the initial stages of the viral life cycle revealing the transcriptionally competent core. These two proteins together form a continuous layer that covers the inner capsid.

The outer capsid consists of 180 copies of the 110-kDa 'sail-shaped' VP2 protein, arranged as 'triskelion' structures (60 trimers) and 360 copies of an interdispersed and underlying globular VP5 protein (59 kDa), arranged as 120 trimers. The VP5 are located more internally than the VP2 spikes and both VP2 and VP5 interact extensively with VP7 protein (T13).

The VP2 protein, responsible for receptor binding⁵³, is positioned on the top surface of the VP7 trimers, while the VP5 globular protein, involved in cell penetration during the initial stages of infection⁵⁴, makes contact mainly with the sides of the VP7 trimers.

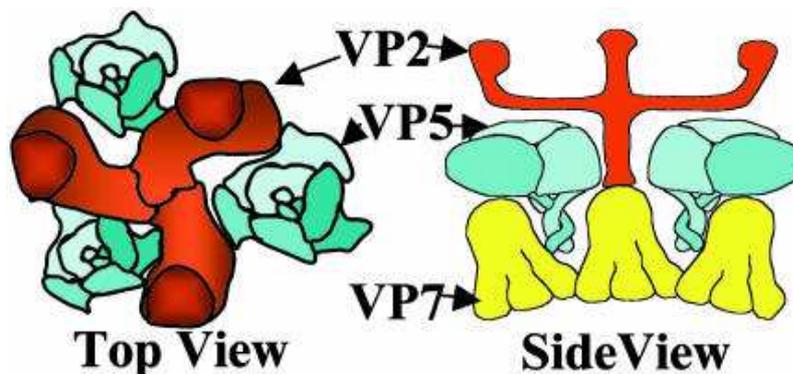


Figure 1.5 Cartoon showing the arrangement of outer capsid proteins VP2 and VP5 with inner capsid protein VP7⁵⁴.

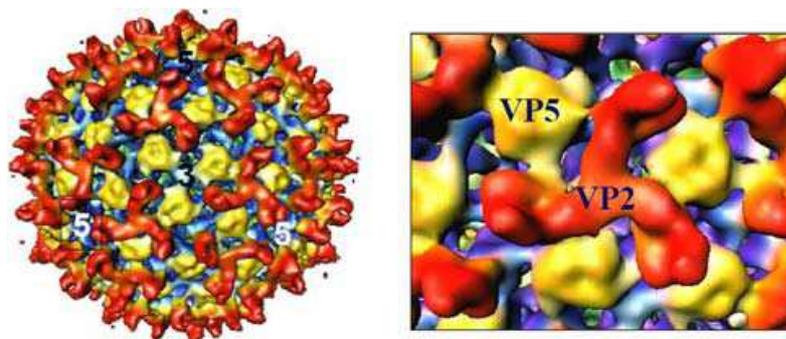


Figure 1.6 Surface representations of the 3D cryo-EM structure (22Å resolution) of BTV. Right: Whole particle with 820 Å diameter showing sail-shaped triskelion propellers (VP2 trimers) in red and globular domains (VP5 trimers) in yellow. Left: A cut-off section enlarged to show the detail of VP2 and VP5 organizations in relation to each other⁶⁶.

VP2 is the most external part of the outer capsid with a propeller-shaped triskelion motif. Each blade of the propeller is ≈ 75 Å in length and 28 Å wide. At the tip of each blade, the molecule broadens to a width of 60 Å and, at this point, bends upwards perpendicular to the plane of the virus. These bent tips give the entire virion a diameter of ≈ 880 Å and extend from the main body of the particle by 30 Å⁵². VP2 triskelion is assembled from three 110-kDa VP2 monomers, each contributing a hub domain and a tip domain.

Each VP2 monomer's hub domain interacts extensively with the other two monomer's hub to form the triskelion hub that holds the VP2 triskelion together. The VP5 trimers are situated in the outer capsid above six-membered rings of VP7 trimers in the core surface layer.

The core is a multi-enzyme complex and it is composed of inner capsid⁵⁵ and three enzymatically active minor proteins (VP1, VP4 and VP6) in addition to the ten segments of dsRNA genome. Particularly, the inner capsid is composed of 780 copies of the VP7 protein (38 kDa) arranged as trimers on a T=13 quasi equivalent lattice which form the 'bristly' core surface. This outer VP7(T13) layer cloaks a thin, rather featureless 'subcore' shell⁵⁶ constructed from 120 copies⁵⁷ of the VP3(T2) protein (Mr 100K).

This VP3(T2) subcore can retain its structure if the outer VP7(T13) core layer is lost.

In addition, the VP3(T2) molecules bind RNA. In fact, the virus genome is composed from 10 segments of dsRNA⁵⁸ and subcore layer, surrounds these segments. Particularly, genomic RNA, is packaged as a series of ordered concentric shells within the VP3(T2) layer of the subcore. The VP3(T2) molecule therefore seems to play a fundamental role in the early stages of formation of the BTV core.

The internal region of the core is occupied by three 'minor' structural protein, VP1, VP4, and VP6, which are associated with the dsRNA genome of 10 segments. These proteins have enzymatic activity and compose the "transcriptase complexes" (TCs).

The minor proteins are attached as a transcriptase complex (TC), to the inner surface of the subcore layer [VP3(T2)] at the 5-fold symmetry axes (at the vertices of the icosahedrons). However, because there is only a single TC at each position they do not have full icosahedral symmetry and it has not yet been possible to determine their organization at the atomic level.

The volume of the core interior was calculated at $60.6 \times 10^6 \text{ \AA}^3$. The volume occupied by the protein components of the TCs was estimated, based on the copy numbers (VP1,12; VP4,24 and VP6,27) and molecular masses (VP1, 150 kDa; VP4, 764 kDa; VP6 35.8 kDa) of the components, as $7.6 \times 10^6 \text{ \AA}^3$. The residual volume for the RNA is therefore $53.9 \times 10^6 \text{ \AA}^3$.

Particularly the VP1 (Pol) is a protein of 150 kDa, is the RNA-dependent RNA polymerase (RdRp), is an essential protein encoded by all RNA viruses that replicate their genome via an RNA intermediate. The second largest minor protein is VP4 and is mRNA capping enzyme in fact is definite like VP4 'Cap' protein. In fact a fundamental feature of eukaryotic mRNAs is that they are modified co-transcriptionally by addition of a 'cap' at their 5' end.

The cap is essentially a methyl-guanosine connected via a 5'-5' triphosphate linkage to the first nucleoside of the transcript (as Mtr 1 that forming the 7-methyl guanosine of the cap structure and Mtr 2 that forming the 2-O-methylguanosine, as the terminal nucleotide of the RNA chain). The cap structure stabilizes the newly synthesized mRNAs and allows efficient translation initiation of mRNAs⁵⁹.

VP6/VP6a (Hel) are protein of 36 kDa and is a RNA helicase protein in fact it has high proportion of hydrophilic and positively charged residues and binds both ssRNA and dsRNA.

Although no lipid envelop is present on mature virions, they can leave the host cell by budding, through the cell plasma membrane. During this process they transiently acquire an unstable membrane envelope.

There are 3 non structural proteins: NS1, NS2, NS3, and NS3A^{48,49}. The 64 kDa NS1(TuP) protein forms tubules that vary in length, up to 4µm, which are of unknown function but regarded as a characteristic feature of orbivirus replication.

The 41kDa NS2(ViP) protein can be phosphorylated and is an important component of the matrix of viral inclusion body (VIB), which are the site of virus replication and assembly. VIBs also contain relatively large amounts of the site of virus core proteins. NS2(ViP) has ssRNA binding activity, suggesting it has an active role in replication. In conjunction with other virus proteins it is believed to be involved in the recruitment of viral mRNA for encapsidation. The NS3/NS3a proteins are two small non-structural membrane proteins (25 and 24 kDa).

As mentioned Nucleic Acid of BTV is RNA and the genomic RNA represents 12% and 19.5% of the total molecular mass of virus particles or cores, respectively. Within the central space of the subcore, there appears to be an association between the dsRNA molecules and the protein density at the 5-fold axes of symmetry (vertices of the icosahedron), which is thought to represent the transcriptase complexes (TCs). From the 5-fold axis, the RNA, in the outmost layer, appears to spiral away from the 5 fold axes outward around the TC for two turns until it clashes with an icosahedrally related neighbour. At this point it is thought to move inward forming the next concentric shell of RNA.

Particularly, the genome, is composed of 10 linear dsRNA (total size is 19.2 kbp, total Mr of 13.1×10^6) segments that are packaged in exactly equimolar ratios, one of each segment per particle.

The genomic RNAs are named “segment 1 to 10” (Seg1 to 10), in order of increasing electrophoretic mobility in 1% agarose gels and in order of decreasing Mw. For BTVs, the segments migrate as three size classes 3 large (Seg1-3: 3.9-2.8 kbp), 3 medium (Seg4-6: 2.0-1.6 Kbp) and small segments (Seg6-10: 1.2-0.8 Kbp).

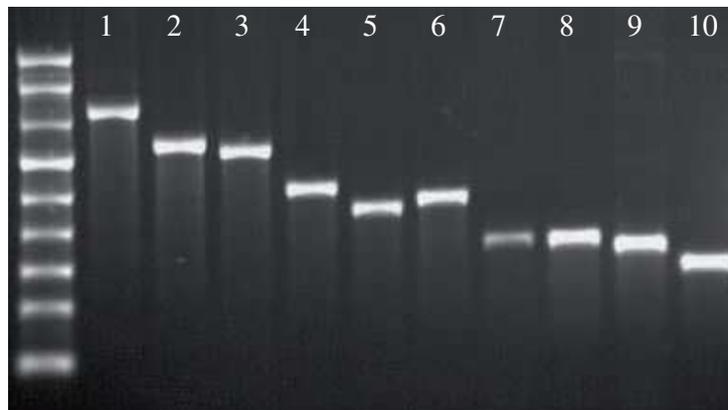


Figure 1.7 Shows the full-length BTV genome segment analyzed by agarose gel electrophoresis. The segments migrate as three size classes: 3 large (Seg1-3: 3.9-2.8 kbp), 3 medium (Seg4-6: 2.0-1.6 Kbp) and small segments (Seg6-10: 1.2-0.8 Kbp)⁶⁰.

The 10 segments forming the viral genome of BTV encode specific viral proteins. In particular: Segment 1 (Seg-1) encodes VP1(Pol); the Seg-2 encodes to VP2; Seg-3 VP3(T2); Seg-4 VP4(Cap); Seg-5 NS1 (TuP); Seg-6 VP5; Seg-7 VP7(T13); Seg-8 NS2 (ViP); Seg-9 VP6/VP6a(Hel); Seg-10 NS3/NS3a.

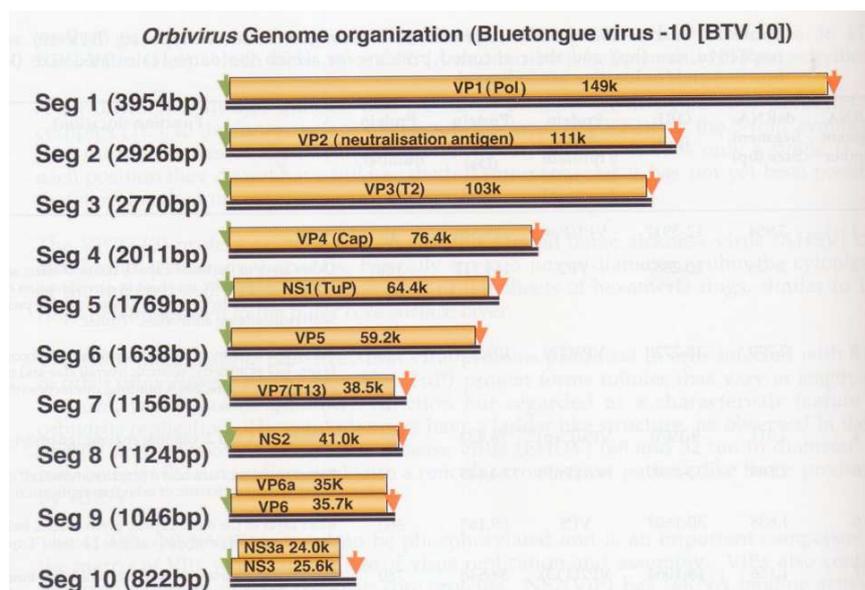


Figure 1.8 Genome organization of Bluetongue virus 10 dsRNA each contain an ORF, except Seg9 and Seg10, which contains two ORFs. The green arrows indicate the upstream conserved terminal sequence (+ve 5'-GuuAAA...), while the red arrows indicate the downstream conserved terminal sequence (+ve ...ACUUAC-3')⁵.

In the genome segments there is only a single major ORF which is always on the same strand (see conserved terminal sequences below).

Only Seg9 and Seg10 contain two ORFs, in fact the Seg9 and Seg10 mRNAs are translated from either of 2 in-frame AUG codons. The significance of the 2 forms of the S9 and S10 gene products (NS3, NS3A; VP6, VP6A) is not known^{61,62}.

The NTRs of BTV and almost all orbivirus genome segments that have been sequenced, contain two conserved bp at either terminus (+ve 5'-GU...AC-3'). The NTRs of BTV include terminal sequences comparable to those of BTVs, but which are not always identical and which may not be conserved in all 10 segments.

The genomic RNA contains 5'-terminal Cap 1 structure (7mGpppG^(2-0m) ...)⁵.

1.2.2 BTV Cycle Replicative

Virus adsorption involves components of the outer capsid. VP2 is responsible for receptor binding: enzymatic treatments of mammalian cells permissive for BTV infection suggest that BTV cellular receptor molecule is a glycoprotein, although it is probable that additional co-receptors are required⁶³. Infact, VP2 structure suggest the presence of two binding site, one on the tip domains of the protein specific for host cell surface glycoprotein, and the other, which appears to bind sialic acid, on the hub domains⁵³.

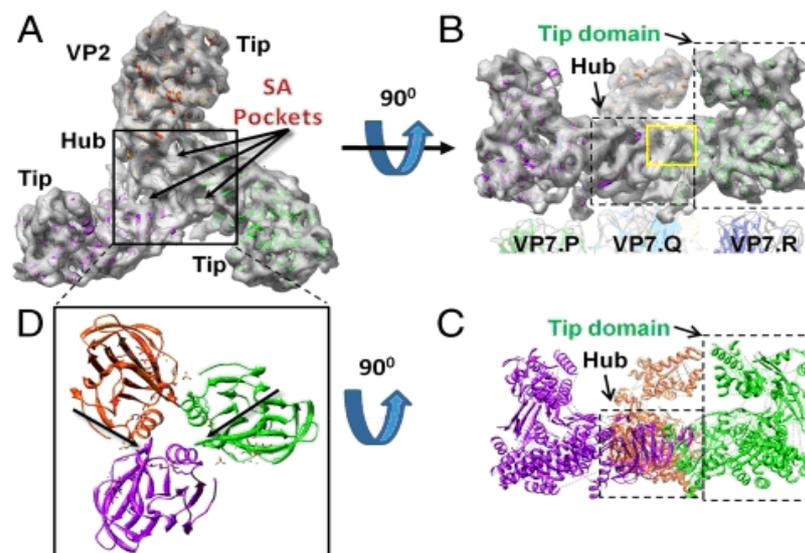


Figure 1.9 Fold of VP2 and identification of SA-binding pocket. Top (A) and side (B) views of the density map of a VP2 triskelion after 3-fold averaging, with three tip domains and a hub (black box) comprised of three hub domains. (C) Ribbon model of (B), rich in α -helices and β -sheets. (D) Top view of atomic-resolution ribbon models of three VP8 SA-binding domains of rotavirus with positions determined by docking to the density map of VP2 of BTV⁵³.

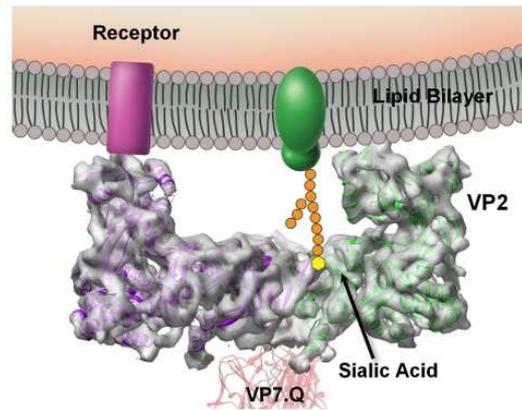


Figure 1.10 Schematic illustration of the membrane attachment of VP2 by its tip domain to a cell surface receptor and by its SA-binding domain to a cell surface glycoprotein⁵³.

During BTV entry into susceptible mammalian cell, VP2 mediates virus attachment to cell and the virus is internalized in clathrin-coated vesicles by receptor-mediated endocytosis. It was observed that with clathrin entry pathway inhibition, using pharmacological and siRNA reagents, virus replication is dramatically inhibited⁶⁴.

After internalisation, the clathrin coat is rapidly lost and vesicle fusion results in formation of an endocytic vesicle (early endosome) within which the outer virus coat is lost (due to low pH within the vesicle). Changes that occur in the outer capsid release amphipathic helices on VP5, which interact with endosomal membrane to initiate permeabilisation, thus releasing the BTV core into host cell cytoplasm⁶⁵. Most BTV particles appear to be converted to core particles and appear in cell cytoplasm within 1-h post-infection⁶⁴.

Upon release into cytoplasm, BTV core particles initiate the transcription of viral genome, and newly synthesized viral messenger-sense RNAs (mRNAs) that are capped but not polyadenylated and are extruded into the cytoplasm.

The two major proteins of the core, VP3 and VP7, account for overall morphology of particle, which marks an end point in virus disassembly and thus protects the viral dsRNA genome from cellular antiviral surveillance mechanisms. The three minor proteins, VP1, VP4 and VP6, constitute a molecular motor that can unwind RNAs and synthesized mRNA molecules.

The largest viral protein, 150 kDa VP1, is the RNA-dependent RNA polymerase (RdRp) with a GDD motifs at positions 763-765 surrounded by the other sequence motifs of polymerase proteins. In the absence of other core components BTV VP1 exhibited little activity. The specificity of the replicase activity for virion 'plus' strands may be determined by other viral core proteins or non-structural proteins, or the specificity may occur during

packaging of the 'plus' strands, without further discrimination occurring during replication. The second possibility seems unlikely⁶⁶.

In any case replication of viral dsRNA occurs in two distinct steps: first, plus-strand RNAs (mRNAs) are transcribed using the negative strand of dsRNA segments as templates, then extruded from the core particle; second, the plus-strand RNAs serve as templates for the synthesis of new minus-strand RNAs at an as yet undefined stage during the assembly of new virus core particles⁶⁷.

The VP1 transcriptional activity needs the VP6 helicase activity. The 59,163 Da VP6 is rich in basic aminoacids (Arg and Lys), has a strong binding affinity for ssRNAs and dsRNAs, has ATP-binding, ATP-hydrolysis, and RNA unwinding activities⁶⁷.

The resulting daughter strand is capped by VP4, which has nucleotide phosphohydrolase, guanylyl transferase and two transmethylase activities required for synthesis of Cap 1 structures⁶⁸.

Bluetongue virus mRNAs are so synthesised exclusively within the viral core and can be directed either into assembling progeny virions within the viral inclusion bodies (VIB) or into the cytoplasm for translation: in turns serves both as template for viral dsRNA genome synthesis and also act as mRNAs for the synthesis of viral proteins within the cytoplasm.

BTV transcripts are not produced at equimolar amounts from the 10 segments: the smaller genome segments are generally the most frequently transcribed, although certain segments (e.g. segment 6 RNA) are synthesized more abundantly than other.

The newly synthesised mRNAs are so extruded from core particles through the channels around the five fold axis of the BTV core, after a distinct conformational change of VP3 and Vp7 proteins core they are released into cytosol.

Newly produced viral proteins later interact with sequestered viral mRNA species within cytoplasmic viral inclusion bodies (VIBs) to form proviral particles. These proviral particles are believed to be the sites of dsRNA synthesis and are considered to be the sites of BTV particle assembly⁵². Viroplasms or 'VIBs' are dense cytoplasmic granular aggregates perinuclear, predominantly consists of the viral coded non structural protein, NS2, which is synthesized abundantly in virus infected cells and is able to bind ssRNA but not dsRNA or DNA⁶⁹.

It's responsible for recruiting both the core proteins and newly synthesized transcripts^{52,70}. This observation is significant since the mechanism by which 10 dsRNA segments that make up the viral genome are selectively recruited and packaged into newly assembling virus particles is unresolved and represents one of the most enduring questions in the

field. Electrophoretic mobility shift competition assays in vitro have suggested that NS2 has a high affinity for specific BTV RNA structures that are unique in each RNA segment⁷¹. Although the RNA binding activity of NS2 can explain how BTV mRNAs are selected from the pool of cellular messages for incorporation into assembling virus particles, it remains unclear how only a single copy of each genome segment is included in newly formed core particles. NS2 could bring the viral RNAs into close proximity, and inter-segment RNA interactions allow the formation of an RNA complex that is the basis of core assembly. Alternately, different RNA subsets that have already interacted may be bound by NS2. The latter hypothesis is consistent with the observations that NS2 may form decameric complexes⁷² and that each NS2 protein subunit may have several RNA-binding domains.

41 kDa NS2 protein is expressed by segment 8, it is highly conserved and is the only BTV phosphoprotein⁷³. Its NS2 expression results in the formation of protein aggregates resembling to VIBs within the cell cytoplasm. Mutation of two NS2 serines (aa 249 and 259) completely inhibits phosphorylation and oligomerisation of the expressed protein, which remains dispersed throughout the cytoplasm⁷⁴.

NS2 is initially dispersed throughout the cytoplasm of BTV-infected cells but rapidly forms into a large number of small discrete cytoplasmic inclusions. Confocal microscopy of BTV-infected cells has shown co-localisation of microtubules and NS2 within early or pre-VIBs⁷⁵. As BTV infection progresses, the VIBs appear to move along tubules to a perinuclear location, becoming larger but fewer in number.

Tubulin has previously been shown to be involved in the formation of ORV VIBs⁷⁶.

Nocodazole, which disrupts tubulin, reduced the number of VIBs in BTV-infected BHK-21 cells. In addition, the VIBs that were produced after such treatment were also randomly distributed within the cell, and none of the larger 'mature' VIBs was observed⁷⁷. Nascent viral particles can often be observed within the matrix of VIBs by EM and fully formed particles are found at their periphery. Fusion of BTV VIBs is believed to occur as their size increases, in some cases trapping areas of cytosol that contain ribosome-like structures and intact BTV virus particles⁷⁸. Early EM studies have indicated the formation of VIBs in association with each infecting 'parental' virus core particle⁷⁹. The exchange of genome segments between different BTV strains that infect the same cell may therefore depend on the fusion of distinct VIBs⁸⁰.

Virus particles are transported within the cell by specific interaction with the cellular cytoskeleton, a network of protein fibres primarily consisting of microfilaments,

intermediate filaments and microtubules within the cell cytoplasm.

The 64 kDa NS1 protein of BTV is synthesized abundantly in virus infected cells and readily assembles as tubular structures within the cytoplasm that are biochemically and morphologically distinct from the microtubules and neurofilaments of normal cells. These high molecular weight tubular structures are formed by helically coiled ribbons of NS1 dimers with a diameter of 52 nm and lengths of up to 1000 nm. The early and abundant synthesis of NS1 and tubules suggests their involvement in virus replication and/or virus translocation.

Structural proteins, with the possible exception of VP6, have inherent capacity to self-assemble into virus-like particles (VLPs) that lack the viral genome⁸¹. The assembly of BTV capsids requires a complex, highly ordered, series of protein—protein interactions. A likely pathway of core assembly is therefore that a number of strong VP7 trimer—VP3 contacts act as multiple equivalent initiation sites and that a second set of weaker interactions then ‘fills the gaps’ to complete the outer layer of the core. This model is possible because not all of the VP7 trimers have equal contacts with the VP3 layer. There is a clear sequential order of trimer attachment onto the VP3 scaffold. The ‘T’ trimers (of the P, Q, R, S and T trimers), which are at the three-fold axes of symmetry, act as nucleation, while ‘P’ trimers that are furthest from the three-fold axis and closer to the five-fold axis, are the last to attach⁸². A VP1/VP4 complex also directly interacts with the VP3 decamer in solution⁸³. By contrast, BTV RNAs, while interacting with the intact VP3 in vitro very efficiently, fail to associate with VP3 decamers under same conditions. The interaction of VP1 and VP4 with VP3 decamers suggests that assembly of the BTV core may initiate with the complex formed by these two proteins and the VP3 decamers, and these assembly intermediates subsequently recruit the viral RNA and VP6 complexes prior to completion of the assembly of the VP3 subcore.

Unlike VP1 and VP4, it has not been possible to confirm the location of VP6 in the core, although it is likely that VP6 is also located within the five-fold axes of the VP3 layer directly beneath the decamer together with VP1 and VP4. VP6 forms defined hexamers in the presence of BTV transcripts and subsequently assembles into distinct ring-like structures.

In addition to binding to viral core proteins, NS2 also binds to viral ssRNA, in fact, as already seen before, it has a role in viral RNA recruitment.

After formation inner capsid and viral RNA incorporation happens viral outer capsid assembly. But the understanding of how this process is controlled is less clear. The certain thing is that the assembly happens between VP2, VP5 and VP7.

It's was observed that addition of VP2 and VP5 abolishes the transcription activity of the core, addition of the outer capsid is likely to be a highly regulated process to prevent premature shutoff of transcription. Unlike rotavirus, there is no evidence that assembly of the outer capsid is associated with the ER. Instead, within BTV-infected cells, virus particles are found associated with the vimentin intermediate filaments.

VP2 alone associates with vimentin intermediate filaments, and disruption of these filaments drastically reduces the normal release of virus particles from infected cells⁸⁴.

In fact the determinants of vimentin localisation are mapped to the N-terminus of the protein and deletions of aminoacids between residues 65 and 114 are shown to disrupt VP2-vimentin association. Site directed mutation also reveals that amino acid residues Gly 70 and Val 72 are important in the VP2-vimentin association. Mutation of these amino acids resulted in a soluble VP2 capable of forming trimeric structures similar to unmodified protein that no longer associated with vimentin. Furthermore, pharmacological disruption of intermediate filaments, either directly or indirectly through the disruption of the microtubule network, inhibited virus release from BTV infected cells⁸⁴.

Therefore the association of mature BTV particles with intermediate filaments are driven by the interaction of VP2 with vimentin and that this interaction contributes to virus egress. Release of newly assembled virus particles varies between host cell types. With insect cells allowing a non-lytic release of virus, while the majority of virus particles in mammalian cells remain cell associated⁸⁵. However, even in mammalian cells that show substantial cytopathic effect in response to BTV infection, the titre of virus in the culture supernatant increases significantly before the onset of dramatic CPE. Thus it is clear that there are defined mechanisms that traffic newly formed virus particles out of infected cells. Virus particles have been observed to leave infected cells in one of two ways.

First, particularly early in infection, particles can be seen to bud through the cell membrane and acquire, least temporarily, an envelope, although evidence for the presence of viral antigen on the outside of such particles is lacking⁷⁹. Second, egress of virus particles from infected cells is accomplished by a process of extrusion in which individual or groups of virus particles move through a local disruption of plasma membrane. This appears to happen without a significant effect on host cell viability, and both types of particles have been observed to be released from some cell surfaces.

Recent evidence has implicated the viral non-structural proteins NS1 and NS3 in this process and has revealed intriguing parallels between the egress of non-enveloped BTV and enveloped viruses⁸⁶. NS3 (229 AA) and its shorter form NS3A (216 AA) are the only membrane proteins encoded by BTV⁸⁷. Both proteins can be found associated with smooth-surfaced, intracellular vesicles⁸⁸ but do not form part of the stable structure of the mature virus.

The NS3/NS3A proteins accumulate to only very low levels in BTV-infected mammalian cells, but in invertebrate cells, the expression levels of these proteins are high⁸⁹. The correlation between high NS3/NS3A expression and non-lytic virus release has suggested a significant functional role for NS3 in virus egress from invertebrate cells, and NS3 and NS3A facilitate the release of baculovirus-expressed VLPs (acting as surrogates for authentic virions) in heterologous insect cells.

The NS3 protein is localized at the site of the membrane where VLPs are released⁸⁸, confirming that the integration of NS3/NS3A into the plasma membrane may trigger the release of mature virions. The cytotoxicity of NS3 requires membrane association of the protein and depends on the presence of the N-terminal transmembrane domain suggesting that NS3 might function as a viroporin and facilitate virus release by inducing membrane permeabilization. It is possible that this permeabilization activity causes local disruption of the plasma membrane allowing virus particles to be extruded through a membrane pore without acquiring a lipid envelope.

The first 13 amino acids of NS3 that are absent in NS3A have the potential to form an amphipathic helix. This cytoplasmic region of NS3 also interacts with the calpactin light chain (p11) of the cellular annexin II complex⁵⁶, a complex that has been implicated in membrane-related events along the endocytic and regulated secretory pathways including the trafficking of vesicles⁹⁰. Although the exact physiological role of this interaction is still unknown, it is likely that interaction of p11 with NS3 may direct NS3 to sites of active cellular exocytosis or that NS3 could become part of an active extrusion process.

There are some indications that cytoskeletal material is released at sites of BTV egress⁹¹, which may be annexin II being drawn through the membrane during the extrusion process, while it is still associated with NS3. The significance of this interaction to BTV egress becomes more apparent in the light of the observation that the other cytoplasmic domain of the protein, situated at the C-terminal end, interacts specifically with the BTV outer capsid protein VP2⁸⁶.

In addition to its interaction with the p11 component of the annexin II complex, NS3 is also

capable of interaction with Tsg101, a cellular protein implicated in the intracellular trafficking and release of a number of enveloped viruses⁹². NS3 recruits the cellular protein Tsg101 to facilitate virus release from mammalian cells and presumably insect cells as well. The ability to usurp the vacuolar protein-sorting pathway is likely to be more important in insect hosts as orbiviruses establish persistent infections in insect cells without causing significant cytopathic effect. Although full clarification of this issue will have to await the availability of a reverse genetics system for BTV, it should be possible to identify insect proteins that interact with NS3 and to shed more light on the question of whether NS3 is better adapted to engage insect proteins, thus facilitating improved virus release. In addition to the inclusion body protein NS2, and NS3/NS3A, BTV produces a third non-structural protein in virus-infected cells.

This protein NS1 is highly conserved between viral serotypes and is synthesized in large amounts, forming up to 25% of total BTV proteins in virus-infected cells. NS1 also forms the tubule structures that are formed in infected cells and are characteristic of orbivirus infections⁹³. Tubules can be seen throughout the infection cycle and as early as 2-4h post-infection, long before the appearance of progeny virus. These tubules appear in groups and are organized in bundles similar to cytoskeletal intermediate filaments. Although the function of NS1 and tubules in the BTV life cycle has been a complete mystery, recent data suggest that the protein is involved in virus morphogenesis.

Besides NS1 is a major determinant of pathogenicity of BTV in the vertebrate host and that its mechanism of action is the augmentation of virus cell association (but not transport of virus to the cell surface), ultimately leading to the lysis of the infected cell.

In summary, it is likely that both NS1/tubules and NS3 are involved in progeny virus maturation and trafficking by acting to facilitate interaction between virus and cellular components.

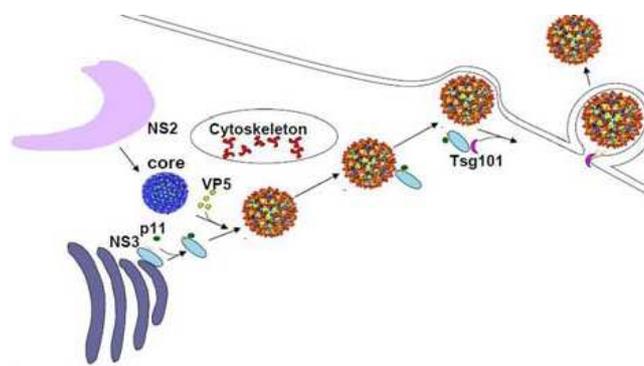


Figure 1.11 Schematics showing the organisation of NS3 protein domains and how NS3 facilitates BTV release. A schematic diagram showing how NS3 may facilitate BTV budding from infected cells. The N-terminus (cytoplasmic) interacts with cellular release factors calpactin p11 and Tsg101 (via late domain motifs) while its C-terminus interacts with the BTV outer capsid protein VP2⁹².

1.3 BTV Disease

1.3.1 Pathogenesis

The pathogenesis of BTV infection is similar in sheep, cattle, and, most probably, in all species of ruminants, although there are marked differences in the severity of disease that occurs in different ruminant species^{12,94}.

After cutaneous instillation of the virus through the bite of a BTV-infected *Culicoides* vector, the virus travels to the regional lymph node where initial replication occurs. From here BTV reaches the secondary replication sites represented by lymphatic tissues (lymph nodes and spleen) and lungs^{12,95,97}. The virus then is disseminated to a variety of tissues throughout the body, where replication occurs principally in mononuclear phagocytic and endothelial cells,^{12,95,97}.

Viraemia is highly cell-associated and is prolonged but not persistent: it is usually detectable around 3-5 days post infection in sheep⁹⁸.

Titres of virus in each cell fraction are proportionated to the numbers of each cell type; specifically, BTV is quantitatively more associated with platelets and erythrocytes and, because of the short lifespan of platelets, BTV is more associated with erythrocytes in the advanced phase of a BTV ruminants infection¹². BTV persist within invaginations in the erythrocyte cell membrane where it is apparently protected from immune clearance⁹⁹, in particular from neutralizing antibodies.

Association of BTV with erythrocytes and mononuclear phagocytes likely facilitates both the prolonged viraemia (that is characteristic of BTV infection of ruminants) and the infection of haematophagous insect vectors that feed on viraemic ruminants.

Furthermore virus localization in mononuclear phagocytes is important for distribution of BTV in the organism.

It has been demonstrated that in advanced phase viraemia is exclusively associated to erythrocytes.

Interestingly, BTV nucleic acid may be detected by polymerase chain reaction (PCR) in the blood of infected cattle (up to 140 days post infection) and sheep (up to 100 days post infection) for many months after it no longer can be detected by virus isolation in cell culture or inoculation of susceptible sheep. Furthermore, ruminant blood that contains BTV nucleic acid as determined by PCR assay, but not infectious BTV as determined by virus isolation, is not infectious to vector insects even by intrathoracic inoculation¹⁰⁰. The viral

determinants of BTV virulence are poorly defined, although there are clear differences in the virulence of individual BTV strains.

This variation can occur as a consequence of genetic drift and/or genetic shift, the latter through reassortment of individual gene segments during infection of animals or insects with more than one virus serotype or strain.

It is logical that this considerable genetic variability of BTV is reflected by differences in phenotypic properties of each virus strain, including their virulence to susceptible ruminants. Thus, whereas strains of BTV serotype 4 apparently cause little or no disease amongst ruminants in the Americas¹⁰¹, a South African strain of the same serotype induced 100% mortality in experimentally infected sheep¹⁰². Serotype, therefore, does not determine virulence.

Similarly, although the vast majority of BTV strains cause little or no disease in cattle, especially in enzootic regions, others like the strain of BTV serotype 8 currently circulating in Europe clearly can induce disease in numerous species including cattle^{103,104}. In contrast, it is proposed that sporadic cases of BT amongst cattle in enzootic regions are the result of an immediate (Type 1) hypersensitivity reaction mediated by IgE¹⁰⁵.

A fundamental question that has vexed scientists for many years is: Why virulent strains of BTV often produce disease in sheep but not in cattle?⁹⁴ The similar or identical pathogenesis of BTV infection of cattle and sheep further emphasizes this obvious paradox.

In fact cattle are asymptomatic reservoir hosts of BTV because they are resistant to developing bluetongue disease, and viremia can persist for several weeks in BTV-infected cattle through a novel interaction of the virus with circulating erythrocytes.

Furthermore, BTV infection of cattle is common in endemic area, thus viremic cattle promote infection of other ruminant species by disseminating BTV to hematophagous *Culicoides* insect vectors¹⁰⁶.

It was recently described^{106,107} inherent differences in the susceptibility of endothelial cells from cattle and sheep to sheep to BTV infection. To facilitate these studies, pure cultures of endothelial cells from the microvasculature of sheep and cattle were isolated and subsequently propagated in vitro, and then their responses to infection with BTV evaluated.

Particularly to better characterize the pathogenesis of bluetongue lung microvascular endothelial cells were selected (primary ovine lung microvascular ECs (OLmVECs)) because pulmonary oedema and microvascular injury are both highly characteristic of BT

and are exposed to BTV and/or inflammatory mediators. After were analyzed the transcriptions of genes encoding a variety of vasoactive and inflammatory mediators.

Interestingly, whereas BTV infection of bovine endothelial cells resulted in endothelial activation, with the increased transcription of genes encoding a variety of vaso-active and inflammatory mediators, and increased expression of cell surface adhesion molecules, similar infection of sheep endothelial cells resulted in minimal activation of endothelial cells.

Particurarly, BTV infection of OLMVECs increased the transcription of genes encoding interleukin-1 (IL-1) and IL-8, but less so IL-6, cyclooxygenase-2, and inducible nitric oxide synthase. In contrast, we previously have shown that transcription of genes encoding all of these same mediators is markedly increased in BTV-infected bovine lung microvascular ECs and BTV infected bovine ECs produce substantially greater quantities of prostacyclin than do sheep ECs.

Thus, sheep and cattle were experimentally infected with BTV to further investigate the role of EC-derived vasoactive mediators in the pathogenesis of bluetongue. The ratio of thromboxane to prostacyclin increased during BTV infection of both sheep and cattle, but was significantly greater in sheep ($p=0.001$). Increases in the ratio of thromboxane to prostacyclin, indicative of enhanced coagulation, coincided with the occurrence of clinical manifestation and activities of EC-derived mediators contribute to the sensitivity of sheep to BTV-induced microvascular injury¹⁰⁷.

Clinical signs and lesions in BTV-infected sheep reflect virus-mediated endothelial injury, as BTV replicates in endothelial cells causing cell injury and necrosis¹⁰⁸.

Moreover, it performs his action in periendothelial cells, in capillar pericytes, in arterioles and venules e capillares.

To replication, follows vacuolar degeneration of affected cells, nuclear and cytoplasmic hypertrophy, pyknosis and karyorrhexis.

For the combined action of cellular necrosis and regenerative processes, are observed hyperplasia and hypertrophy of endothelial cells with consequent vasal occlusion, blood stasis, exudation, associated to hypoxia, up to even fulminant hemorrhage.

It seems that vasal endothelium aggression is selective, that means that is not generalized to all districts of the organism but only to certain body organs or even to particular branches of blood vessels.

1.3.2 Clinical Signs

The vast majority of infections are clinically inapparent. In a percentage of infected sheep and some wild ruminants more severe disease can occur.

In sheep, the severity of disease varies with the breed of sheep, virus strain and environmental stresses. The morbidity rate can be as high as 100% in this species. The mortality rate is usually 0-30%, but can be up to 70% in highly susceptible sheep or as a consequence of highly virulence strains^{4,109}.

The clinical signs are the result of virus-mediated microvascular injury that produces oedema, hyperemia and vascular congestion, haemorrhage and tissue infarction^{4,98}.

The course of the disease can vary from peracute to chronic. Peracute cases usually die within seven to nine days of infection, mainly as a result of lung oedema and eventual asphyxia^{109,110}.

The acute form is the most frequent. The incubation period is about seven days and the disease starts with high fever (41-42°C) persisting for 2-14 days and at the same time occurs a state of malaise and anorexia.^{4,109,110} More frequently, the febrile reaction lasts about six to eight days and its termination is determined by the course of the disease and the extent of secondary infection. Generally there is a fair correlation between the duration of the fever and the severity of the disease¹⁰⁹.

Other clinical signs may appear within one or two days of the onset of fever. There is reddening of the oral mucosa that, after 2 days from the expression of clinical signs, it becomes intense hyperaemia of the tongue, face, neck and auricular skin. Follows an excessive salivation, conjunctival and nasal drainage with respiratory distress. The nasal and ocular discharge are initially serious, then they become increasingly catarrhal, mucopurulent and sometimes hemorrhagic (bloody nasal). This tends to dry, forming crusty exudates accumulated around the nostrils. They also have swelling of the tongue which, in serious cases, becomes cyanotic, hence the name of the disease. Edema occurs simultaneously: lips, intermaxillary region and brisket^{109,110}.

After 5-8 days begin ulcerative processes of the oral mucosa that appears covered with necrotic exudates of fetid odour. The oral erosions and ulcers cause superficial hemorrhages (bleedings), open sores on the tongue, mouth and nostrils and are also cause of food refusal and subsequent dehydration and weight loss. They also induce pain which may cause the animal to submerge its mouth and lips in drinking water for prolonged periods. In the worst cases, with impediment of normal reflux may cause

aspiration of rumen contents which can lead to aspiration pneumonia and death within 48 hours^{109,110}.

Lesions also appear around the base of the claws and coronary band occurring erythematosis with hemorrhagic areas. The coronitis appears around 12-21 days may, usually towards the end of the febrile reaction. Hyperemia of the coronary bands and petechiae under the periople, which later become streaky in appearance as a result of haemorrhage into the fine medullary canals of the growing horn, give rise to a red zone or band in the horn of the hoof. The lesion is most pronounced on the bulbs of the feet, and particularly of the lateral digits. The hind feet are most frequently affected. The feet are warm and painful, and affected animals are reluctant to move and often stand with arched backs or are recumbent. In affected sheep the gait is often stiff with varying degrees of lameness. Sometime severely affected animals try to walk on their knees. In animals that recover, the bands of discolouration in the hooves grow out and a 'break' in the hoof may develop ('slipper formation'), with the old horn eventually sloughing off after three to four months^{109,110}.

Inability to move and recumbency may also be exacerbated by emaciation and muscles lesions. Degeneration and necrosis of skeletal muscles in the neck may lead to torticollis. Hyperaemia of the skin is usually most severe in those areas that are exposed to sunlight (such as, in Merino sheep, the face, ears and legs which are not covered by wool), but may involve the whole body¹⁰⁹.

In most affected animals the wool fibres 'break' within the wool follicle and the fleece may be shed three to six weeks later^{109,110}.

After infection of ewes during early pregnancy can occur abortion, mummified fetal, congenital abnormalities (arthrogryposis, hydranencephaly, ataxia) or give birth to 'dummy' lambs^{109,110}.



Figure 1.12 Gross lesions of bluetongue in sheep. **a)** Extensively crusted nasal mucosa. **b)** Hyperaemia of the oral cavity and oedema of the mucous membranes **c)** The feet are often affected with coronitis and laminitis causing lameness. **d)** loss of wool. (Photo courtesy of FAO)

Most animals that succumb to acute BT die within 14 days of infection, and sheep that survive the acute disease can have a prolonged convalescence and some will show substantial deterioration in body condition and become emaciated. Recrudescence of clinical disease has been reported.

Sometime, the disease evolves in sub-acute forme. There is emaciation, weakness and stiff neck followed by a long period of convalescence.

It is observed, in light BTV forms, a transitory fever rise. BTV-induced muscle injury and necrosis prevent normal locomotion in some animals and can lead to torticollis. The wool of convalescent sheep is frequently shed, and some sheep shed their as bacterial pneumonia.

Although infections in cattle and goats are usually subclinical, the severity of the disease strongly depends on the BTV serotype; BTV-8 is proved to be particularly aggressive in cattle and goats too, causing a disease similar in severity to those of sheep.

More frequently, the only signs of disease are changes in the leukocyte count and a fluctuation in rectal temperature. Rarely, cattle have mild hyperemia, vesicles or ulcers in the mouth; hyperemia around the coronary band; hyperesthesia; or a vesicular and ulcerative dermatitis. The skin may develop thick folds, particularly in the cervical region. The external nares may contain erosions and a crusty exudate. Temporary sterility may be seen in bulls. Infected cows can give birth to calves with hydranencephaly or cerebral cysts. Cattle that have clinically apparent disease may develop severe breaks in the hooves several weeks after infection; such breaks are usually followed by foot rot.

1.3.3 Lesions

The lesions reflect congestion, oedema and haemorrhage as a consequence of virus-mediated microvascular injury.

The face and ears are often edematous. Oral erosions and ulcers and bloody nasal discharge with crusting around the nares may be seen. The coronary bands of the hooves are often hyperemic; petechial or ecchymotic hemorrhages may be present and extend down the horn.

Lesions present on post-mortem examination of affected sheep can include hyperaemia, haemorrhages, erosion and ulceration of the mucosa of the upper gastrointestinal tract (oral cavity, oesophagus, forestomachs); petechiae, ulcers and erosions are common in

the oral cavity, particularly on the tongue and dental pad, and the oral mucous membranes may be necrotic or cyanotic. Hyperemia and occasional erosions may be seen in the reticulum and omasum. The nasal mucosa and pharynx may be edematous or cyanotic, and the trachea hyperemic and congested; pulmonary oedema. Petechiae, ecchymoses and necrotic foci may be found in the heart., with the papillary muscle of the left ventricle being an especially characteristic site. Subintimal haemorrhages in the pulmonary artery and the hemorrhage at the base of the pulmonary artery is particularly characteristic of this disease. Pleural and/or pericardial effusion. Oedema and haemorrhage of lymph nodes. Facial and submandibular oedema. Finally, the skeletal muscles may have focal hemorrhages or necrosis, and the intermuscular fascial planes may be expanded by edema fluid⁴.

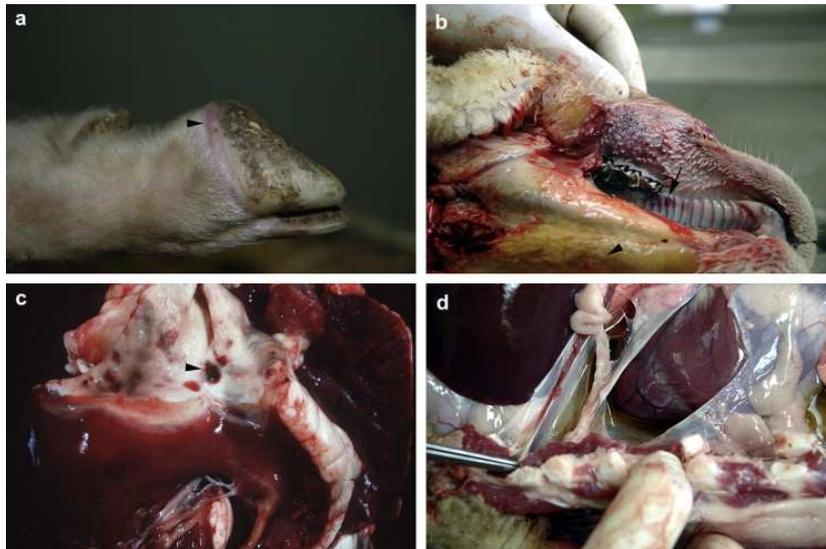


Figure 1.13 Gross lesions in sheep with bluetongue. (a) Distal limb: coronitis (arrowhead). (b) Head: submandibular oedema (arrowhead) and haemorrhage and ulceration (arrow) of the hard palate and dental pad. (c) Heart: subintimal haemorrhages (arrowhead) in the pulmonary artery. (d) Pericardial space: pericardial effusion⁴.

Histological lesions reflect endothelial hypertrophy, vascular stasis and thrombosis with tissue infarction. Specifically, lesions in skeletal and cardiac muscle range from acute myonecrosis with haemorrhage, to more chronic lesions with fibrosis and infiltration of mononuclear inflammatory cells. Pulmonary oedema is very characteristic of many fatal

BTV infections, but certainly is not pathognomonic of BT. Changes within small vessels in the skin and adjacent to lesions such as oral ulcers are inconsistent and often subtle; acutely affected vessels may exhibit only endothelial hypertrophy with perivascular oedema and/or haemorrhage, with subsequent variable perivascular accumulation of lymphocytes and macrophages⁴.

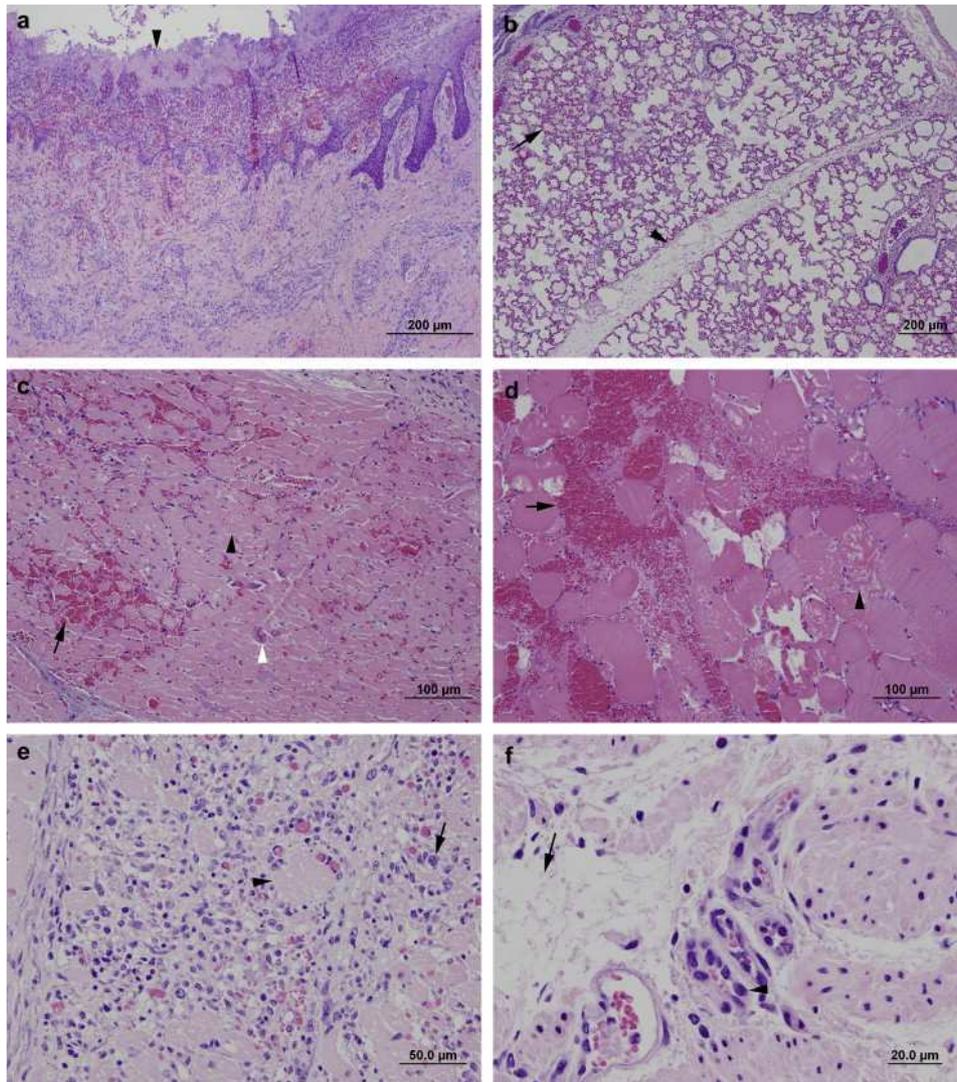


Figura 1.14 Histopathological lesions in sheep with bluetongue. (a) Buccal mucosa: superficial epithelial ulceration/erosion (arrowhead) with submucosal haemorrhage and inflammation. (b) Lung: pulmonary oedema, with expansion of the interlobular septae (arrowhead) and accumulation of protein rich fluid in airspaces (arrow). (c) Heart: acute myocardial necrosis (arrowhead), haemorrhage (arrow) and mineralization (white arrowhead). (d) Skeletal muscle: acute myonecrosis (arrowhead) and haemorrhage (arrow). (e) Heart: myonecrosis (arrowhead) with mononuclear inflammation (arrow). (f) Pulmonary artery: endothelial hypertrophy (arrowhead) and perivascular oedema (arrow)⁴.

1.4 BTV Proteins Expression System

The predominant serodiagnostic assay used in many countries to detect bluetongue virus infections is a competitive enzyme-linked immunosorbent assay (c-ELISA) which employs two critical reagents: a cell culture-derived BTV antigen and group-specific monoclonal

antibody (Mab). Previous studies demonstrated that native VP7 protein could be used for BTV serodiagnosis due to its ability to react with group-specific antibodies^{111,112}, but ongoing difficulties have been reported by laboratories in the production and quality control of the native antigen reagent which relies on the presence of adequate molar quantities and appropriate presentation of the major BTV core protein VP7.

Recombinant techniques have been used to clone the gene (coding for the VP7 protein) and to express it in a variety of systems to produce a stable reagent for the use in serodiagnostic assays such as ELISA. The variable performance of recombinant VP7 reagents in different ELISA formats can be attributed to the inherent properties of a particular expression system and purification procedure. For example, a yeast-expressed BTV-1 VP7 recombinant antigen when used in a blocking ELISA required the test monoclonal antibody (Mab) to be 30 times more concentrated than when it was used with native virus antigen¹¹³. This result indicated that VP7 protein conformation was an important factor respect to antigenicity and was further confirmed by epitope mapping studies¹¹⁴. High-yield production of recombinant VP7, which was structurally and antigenically equivalent to native VP7, has been demonstrated using a baculovirus expression system¹¹⁵. Due to difficulties encountered in the production of consistent batches of native VP7-containing preparations for the use in serodiagnostic assays, baculovirus expression system was selected for the production of a BTV rVP7 protein. Insect cells have been shown to carry out many types of post-translational modifications, which influence protein structure. Baculovirus expressed recombinant proteins including analogous proteins from other *Orbiviruses*^{115,116,117}, this proteins have been demonstrated to be antigenically, immunologically and functionally similar to their authentic counterparts¹¹⁸. The high yield production of viral proteins from this system has offered new ways to study viral protein function and to develop diagnostic tests.

In a Luo and Sabara paper, a recombinant baculovirus was constructed containing a cDNA copy of genome segment 7 of BTV serotype 11 and used to infect insect cells, *SF21* (*Spodoptera frugiperda*), which, in turn, expressed high levels of VP7 protein with an estimated molecular mass of 39 kDa. In its purified form, this recombinant protein could be detected by group-specific Mabs designated 3.17.A3 and 8A3B.6 produced against BTV serotypes 1 and 17, respectively, as well as by polyclonal bovine antibodies raised against North American and South African BTV serotypes. No reactivity was observed by Western blot analysis with these two Mabs suggesting that the common antigenic determinants, on BTV VP7 protein, were mainly conformational. Due to its antigenic similarity to native

antigen, the recombinant protein was found to be a suitable replacement for the use in c-ELISA to detect BTV-specific antibodies with the added advantage that it could be consistently produced and was, therefore, amenable to quality control testing for purity, stability and other standards .

Even greater difficulties are found in vaccine production. First, BTV exists as 24 distinct serotypes at least, and protection afforded by vaccines is serotype specific. Thus, an animal vaccinated and protected from infection with one serotype of bluetongue is not protected from subsequent infections with other viral serotypes. Also, reassortment of genome segments in hosts co-infected with more than one strain of BTV can readily lead to progeny strains with a mixture of the characteristics of the parental strains and possibly to new serotypes. Only two vaccine types for bluetongue are currently available commercially, live attenuated vaccines and inactivated virus vaccines, and although both of these options provide robust protection, they are not without significant drawbacks. The first are linked to abortion and teratological effects in vaccinated animals¹¹⁹; reassortment between strains, including vaccine strains, has led to emergence of reassortant field strains of the virus¹²⁰; finally, could potentially also be transmissible by biting midges and, most importantly, do not distinguish between infected and vaccinated animals. The second, inactivated by treating BTV with beta-propiolactone, gamma radiation or binary ethylenimine, have demonstrated safety and good immunogenicity with 2 doses of bivalent vaccine 4 weeks apart, but it is important that the virus be completely inactivated in every vaccine batch, as otherwise vaccination could lead to some of the problems discussed above for attenuated vaccines.

In addition to attenuated live virus and inactivated virus vaccines for bluetongue, there are a number of other viable alternatives that promise to address the unmet requirements for an effective bluetongue vaccine: low cost, ability to distinguish between vaccinated and infected animals, broad protective immunity against multiple serotypes and, preferably, a single dose. Since, as is well known, VP2 protein of BTV alone was sufficient to elicit protective immune responses in sheep, combining both virus outer capsid proteins (VP2+VP5) resulted in better protection than VP2 alone. A range of different approaches, including purified recombinant protein, canarypox-vectored expression, and capripox-vectored expression, are currently tested.

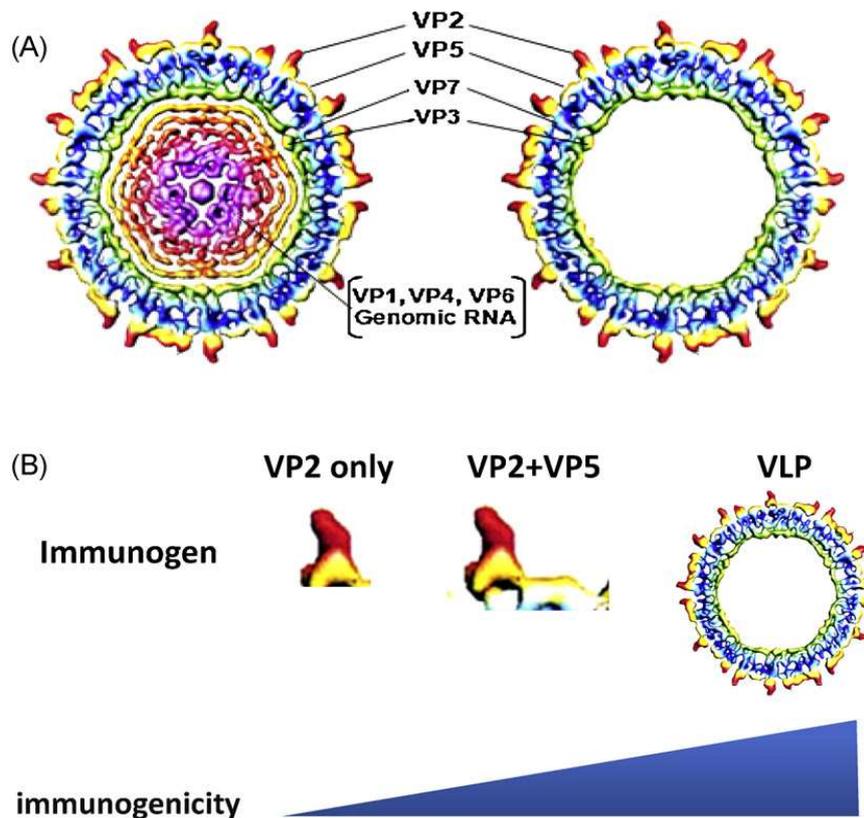


Figure 1.15 Increase in immunogenicity achieved by combining bluetongue proteins in a single vaccine. (A) Left: Cryo-EM reconstruction of a section through a bluetongue virus particle; Right: a reconstruction showing the components of the full particle that are present in VLPs. (B) An indication of the immunogenicity of bluetongue proteins when used as subunit vaccines¹¹⁹.

For poxvirus-based systems, poxvirus is used as a vehicle for BTV genes transfer into sheep cells, where BTV proteins are then synthesized. Perhaps unsurprisingly, given the initial observation, the vaccination of sheep with canarypox vector (a ubiquitous vector unable to replicate in mammalian cells that co-expressed VP2 and VP5) elicited neutralizing antibody and protected sheep against challenge with BTV¹²². A further alternative approach used a capripox vector to express VP2, VP7, and the non-structural proteins NS1 and NS3, all of which partially protected sheep from the disease¹²³. Although these vaccines have generally been effective when tested in sheeps and other ruminants, it should be noted that capripox vaccine was only partially protective, suggesting that not only the protein expressed protein but also the amount produced and its availability to interact with the immune system may be crucial. Most importantly, poxvirus-vectored vaccines, that express a small fraction of the BTV genes, have no risk of acting as a source of virulent BTV, but, containing both the protein and the nucleic acid, retain the theoretical potential to recombine with field strains.

One option that has been pioneered for the recombinant protein approach for BTV vaccines is the assembly of Virus-like particles (VLPs).

VLPs for BTV are produced in insect cell culture of *Spodoptera frugiperda* SF9 or SF21 and *Trichoplusia ni* BTI 5B1-4^{124,125,126}, using a baculovirus-based protein expression system. This eukaryotic expression system can produce large amounts of protein more efficiently than mammalian cell expression systems, it can fold and assemble proteins and large complexes (a single BTV VLP has a molecular mass of ~83.8 mDa) and it uses an environmentally disabled form of an insect virus that is easily inactivated to drive protein expression.

2. AIM OF WORK

Bluetongue is an important disease of economic relevance for the livestock industry, and an efficient diagnosis and an effective prophylactic immunization of sensitive animals are the most practical measures to combat BTV infections. In order to have standardized, specific and sensitive serodiagnostic assay and successful prophylactic immunization plans, is essential to investigate new molecular tools.

VP2, the outermost viral protein, is the cellular binding protein, elicits virus-neutralizing antibody, and is responsible for hemagglutinin activity and serotype specificity; thus, VP2 is one of the most studied viral protein.

VP2 has been expressed successfully through many systems and, currently, the insect cell-baculovirus expression system is the most effective platform to rapidly produce high levels of recombinant BTV proteins. Insect cells have been shown to carry out many types of post-translation modifications (PTM), which influence protein structure and conformation, and baculovirus expressed BTV recombinant proteins have been demonstrated to be antigenically, immunologically and functionally similar to their authentic counterparts.

However, mammalian cell expression systems are preferred for the production of large and complex proteins requiring PTM the most similar possible to those natural occurring in field infection.

Due to its biochemical characteristics, VP2 is a complex protein proven to be difficult to express as a soluble form in mammalian cells. Therefore obtaining VP2 secreted into the medium of stably and transiently transfected mammalian cells represents a challenge.

In the present work we investigated a mammalian expression platform for BTV-8 VP2 production in a soluble form.

3. MATERIALS AND METHODS

3.1 Polymerase Chain Reaction

PCR is an extremely simple and versatile technique for amplification and revealing of nucleic acids fragment.

For PCR reaction uses two primers with new sequences, that in this case, contain two new restriction sites. PCR cycles are performed on a plasmid template to insert in frame new restriction sites, that will be found in the ends.

The exact sequences of the regions flanking the desired fragment are necessary in order to apply this technique: with this information a couple of primers can be designed, assuring the specificity of the reaction.

Primers are oligonucleotides with variable length: each one of them pairs with one of the strand of template DNA, at both ends of the region which is to be amplified. A DNA polymerase is used to extend these primers in various cycles of DNA denaturation, primers annealing and extension.

Because nucleic acids denaturation step is carried out at 94 °C and the extension one at 72 °C, DNA polymerase must be thermostable and it must work at these temperatures. For this reasons enzymes of thermophilic organisms are used, like thermostable polymerase from *Thermophilus aquaticus* (*Taq*) or *Phyrococcus furiosus* (*Pfu*). *Taq* is error prone, since it lacks a 3'→5' exonuclease activity, and error rate is between one mismatch on 10⁴ nucleotides incorporated to two on 10⁵.

Here components of a typical PCR mix are listed, with their respective concentrations:

- 0.2 mM dNTPs mix;
- 0.25 µM primers;
- 100 ng –1 µg template DNA;
- 1 U *Taq* polymerase (Fermentas);
- Buffer 1X pH 8.3 (Fermentas); Buffer composition is: 10 mM Tris-HCl, 2.5 mM MgCl₂, 50 mM KCl.
- Sterile water till 50 µl.

Another important aspect that is to be considered for the success of a PCR is the use of correct parameters: every cycle comprises three phases that are template denaturation at 94 °C for 1 minute, primers annealing and primers extension at 72 °C. Temperature of the

annealing step varies depending on primers bases composition: usually it is set at 5-10°C lower than primers melting temperature in order to have high specificity binding of the primers to the template.

Primers melting point depends on oligonucleotides length and their GC content: the longer they are and the higher GC rate is, the higher denaturation temperature is. This temperature can be estimated with a mathematical formula:

$$T_m = 4x(GC)+2x(AT)$$

3.2 Agarose gel electrophoresis

Agarose gel electrophoresis technique is a separation method widely used in molecular biology to separate and identify nucleic acids fragments of different lengths.

At pH 7 phosphate groups are not protoned, so DNA molecules are negatively charged and it is possible to make them migrate in an agarose gel towards the positive charge of an electric field (anode). Because of their various size, DNA fragments migrate with different speed: the bigger the fragment size, the higher the resistance met in moving through the gel.

Agarose is a complex polysaccharide and it is extracted from red algae; it is constituted by D-galactose and anhydrous 3,6 L-galactose units.

Agarose is soluble in boiling water and it forms a gel when cooling, by the generation of hydrogen bonds between the polymeric chains.

The basic principle is a molecular sieve through which the molecules should pass. As for DNA molecules the unitary charge is constant, the only discrimination occurs among different dimension: larger molecules are retained from the gel's meshes and the smaller result faster in their ride toward anode.

DNA is loaded in wells made out of the gel together with a proper buffer that contains glycerol (that increases the sample density and facilitate its loading) and a dye (that allows us to see how much DNA ran).

In order to view DNA bands after the run, ethidium bromide is added during preparation of the gel at a concentration of 0,1 µg/ml. This molecule is hardly fluorescent in pure solution, but if DNA is present, it can insert itself between base pairs: the resulting complex has a

much higher fluorescence, allowing viewing of DNA fragments during exposure to ultraviolet rays.

In order to have some information on DNA fragment size, a reference sample (ladder DNA) can be loaded in a well: these samples consist in a solution of oligonucleotides of various known length.

Quantified ladder DNA solution are also available: not only fragment size is known, but also DNA concentration for each band. By comparing the fluorescence emitted by the sample and the one emitted by the reference, it is possible to approximate DNA concentration in the sample.

3.3 DNA extraction from an agarose gel

Once DNA fragments have been separated through electrophoresis, it is possible to recover the interested ones using a suitable commercial kit.

To avoid contamination it is necessary that fragment bands are well separated and the cut should be done as close as possible to the band.

The same reagents, columns and protocol can be used to purify PCR products.

Here it is presented the protocol for "EuroGold Gel Extraction Kit ", produced by Euroclone.

Protocol DNA extraction gel:

1. Excise the agarose slice from the gel and weigh it.
2. Put the gel in a 1.5 ml microfuge tube and add 100 µl of Solubilisation buffer XP2 for every 100 mg of gel (solubilisation buffer contains concentrated NaClO₄, sodium acetate and TBE solubilizer).
3. Incubate at 40°C for at least 10 minutes or until gel has completely dissolved; monitor the pH value of the mixture after the gel is completely dissolved, because DNA yield will be significantly decreased when pH value is >8. The colour of the mixture should be light yellow, if it becomes orange or red, add 5 µl of sodium acetate to bring the pH down.
4. Put the spin column in a 2 ml test tube.
5. Transfer the sample in the column, 750 µl at a time, and centrifuge at 10.000 x g in a microfuge for 1 minute in order to make DNA bind to the column.
6. Discard the flow-through and place back the column in the test tube.

7. In order to wash the column, add 750 μ l of SPW wash buffer (containing ethanol, NaCl, EDTA and Tris/HCl) and centrifuge for 1 minute at 10.000 x g.
8. Repeat step 7 once more.
9. Discard the flow-through and centrifuge for 3 minutes at maximum speed. This additional drying step is required to remove all traces of ethanol, which eventually could inhibit subsequent enzymatic reactions.
10. Transfer the column in a clean 1.5 ml test tube.
11. To elute DNA, add 50 μ l of sterile filtered water directly on the centre of the silica matrix and centrifuge at 5.000 x g for 1 minute.

3.4 Cell cultures

Animal cell lines are grown in plastic flasks: cells form a monolayer which is tightly adherent to the bottom. They replicate until they reach confluence, until they completely cover the internal surface of the flask.

If non-neoplastic cell lines are used, contact inhibition remarkably reduces the growth rate at this point, so it is necessary to transfer the culture to a bigger flask.

Alternatively the cells can be split to more flasks: the number depends on the amount of cells and on their growth rate.

Cells are grown in EMEM (Eagle Minimum Essential Medium, Lonza) with 10% FCS (Foetal Calf Serum), 2mM L-glutamine, 100 IU/ml penicillin (SIGMA), 100 μ g/ml of streptomycin (SIGMA) and 2.5 μ g/ml of Amphotericin B (SIGMA).

For this research were used Human Embryo Kidney (HEK) 293T cell lines : these lines have a very high growth rate and a epithelial morphology. HEK 293T contains, in addition, the SV40 Large T-antigen, that allows for episomal replication of transfected plasmids containing the SV40 origin of replication. This allows for amplification of transfected plasmids and extended temporal expression of the desired gene products.

Passage of cells from one flask to another involves proteolytic enzymes which degrade membrane proteins such as cadherins, responsible of cell-cell interaction, and integrins, involved in cell-flask interaction. A wide variety of enzymes are available, depending on the cell type that is to be treated: one of the most used is the protease trypsin.

Prolonged exposure to trypsin may result in cell death instead of the mere detaching from the wall of the flask: the amount of protease and the time of exposure depends on the characteristics of the cell line.

Cell culture passage protocol:

1. remove the medium from the flask but not completely .
2. Wash the monolayer with trypsin (3 ml for a 25 cm² flask, 10 ml for 75 and 150 cm² ones). This washing step must be short, because its only purpose is to remove the remaining traces of debris.
3. Remove trypsin. At this stage monolayer should be visible as an opaque coating on the bottom of the flask.
4. Add a suitable amount of trypsin to detach the cells (0.5 ml for 25 cm² flask, 2 ml for 75 ones and 4 ml for 150 ones).
5. Shake the flask until complete detaching of cells: anyway this procedure should not last more than few minutes in order to avoid flocculation and cell death; if the cell line produces a lot of extracellular matrix, to completely detach cell from plastic flask, incubation at 37°C with trypsin may be required.
6. Neutralize trypsin by adding fresh medium with 10% FCS: usually 2 to 5 ml are sufficient.
7. Wash the flask wall to recover as many cells as possible.
8. Split cell suspension to new flasks.
9. Add to every flask an adequate amount of medium (about 10 ml for a 25 cm² flask, 20-25 ml for a 75 cm² flask, 30-40 ml for a 150 one).
10. Incubate the flask at 37°C in an environment with 5% CO₂.

3.5 Electroporation

DNA molecules, like other polar ones, are unable to pass freely through the membrane, largely composed by lipids which self assemble in a phospholipids bilayer.

Electroporation has been developed to introduce polar molecules in a host cell through the cell membrane; a temporary large electric pulse disturbs the bilayer causing the formation of temporary aqueous pores, allowing polar molecules to pass through the plasma membrane.

The overall efficiency of this technique is higher than other methods like calcium phosphate precipitation even if about half of the cells dies because of the electric shock during the procedure. If the pulses are wrong in intensity or on length, some pores may become too large and fail to close after membrane discharging, causing irreversible cell damage; furthermore, material transport inside and outside the cell, that is relatively

unspecific during electropermeability, may result in ion imbalance and consequently cell death.

Because of the different characteristics of eukaryotic and prokaryotic cells, protocols for electroporation vary in few aspects that are however of primary importance for the outcome of the transformation. They depend both on the presence of a cell wall in bacteria and on completely different culture conditions.

Generation of electrocompetent bacteria (DH10B or HS996):

1. Thaw out an aliquot of bacteria from a stock culture at -80°C and leave it on ice.
2. Inoculate 500 μl of cells in 50 ml of Luria Bertani medium (LB) and leave overnight at 37°C on a stirrer. A control should be done adding 500 μl of cells in 50 ml of LB with 20 $\mu\text{g/ml}$ of cloramphenicol.
3. Transfer 10 ml of the confluent culture in 300 ml of fresh LB without antibiotics, leave at 37°C on a stirrer until O.D._{600} (measured at 600 nm) is about 0.5-0.6.
4. Cool bacteria culture in ice and centrifuge at 7000 *rpm* at 0°C for 5 minutes.
5. Discard the supernatant and resuspend pellet in 50 ml of cooled sterile water.
6. Centrifuge at 7000 *rpm* at 0°C for 5 minutes.
7. Repeat step 5 and 6.
8. Discard the supernatant and resuspend pellet in 50 ml cooled sterile water with 10% glycerol.
9. Centrifuge at 7000 *rpm* at 0°C for 10 minutes.
10. Discard the supernatant and resuspend pellet in 1ml of cooled sterile water with 10% glycerol.
11. Make aliquots of 50 μl of cells in PCR vials; cool vials in a liquid Nitrogen bath and store at -80°C until used.

Electrocompetent cells have been prepared as described in Sambrook and Russel¹²⁷.

Electrocompetent bacteria transformation protocol:

1. Thaw out an aliquot of electrocompetent bacteria from a stock culture at -80°C and leave it on ice.
2. Add the DNA solution to the test tube (1 or 2 μl , depending on plasmid concentration) and mix thoroughly with a pipette.
3. Transfer the suspension to an electroporation cuvette previously cooled on ice.

4. Put the cuvette in the electroporator (Equibio EasyJet Plus Apparatus) and start the electrical discharge.
5. The parameters used are: 2500 Volts, 25 μ Farad, 201 Ohm. In case of high impedance add 1 μ l of diluted PBS.
6. Immediately add 1 ml of LB medium. It is very important to quickly add the medium after the discharge in order to reduce its toxic effect on the cells.
7. Transfer bacteria to a 15 ml test tube and incubate it on an agitator at 37°C for 1 hour in order to make bacteria recover and express the selectable marker that is in the plasmid.
8. Plate 250 μ l of bacterial suspension on selective solid medium and incubate the petri dish at 37°C overnight.

Animal cells electroporation protocol:

1. Collect the cells from a confluent flask through trypsinization.
2. If an exact number of cells should be electroporated, count them with a Burker chamber and put the necessary amount of cells in a 15 ml test tube.
3. Centrifuge at 180 x g (1000 *rpm*) for 2 minutes to pellet cells.
4. Remove the culture medium, wash pellet with 700 μ l of DMEM-high (Dulbecco's Eagle Modified Minimal Essential Medium, with high percentage of glutamine and pyruvate) with 20 μ g /ml of gentamicyn but without FCS .
5. Centrifuge at 180 x g (1000 *rpm*) for 1 minute to wash cells; resuspend the cells with 500 μ l of DMEM without FCS.
6. Add the desired amount of DNA solution to the suspension and transfer the content of the test tube to an electroporation cuvette.
7. Put the cuvette in the electroporator and start the electrical discharge. Parameters may differ according to the cell lines employed; for BEK and for HEK cell line these parameters were used: 270 Volts, 1500 μ Farad or 186 Vols, 1500 μ Farad.
8. Add as quickly as possible 1 ml of MEM with 10% FCS.
9. Transfer the suspension to a new culture flask (25 cm² or 75 cm² flask, according to the starting amount of cells) and add 10-15 ml of MEM with 10% FCS.
10. Incubate at 37°C in a 5% CO₂ atmosphere for 1 hour or at least until cells start to adhere to the flask.
11. Replace medium and incubate again at 37°C in a 5% CO₂ atmosphere.

3.6 Heat shock

Heat shock is another procedure used to introduce foreign DNA in bacterial cells.

If followed scrupulously, Hanahan's procedure can reproducibly generate competent cultures of E.coli that can be transformed at high frequencies (5×10^8 transformed colonies / μg of plasmid DNA)¹²⁷.

Hanahan Transformation of XL1Blue:

1. Inoculate LB with an aliquot of XL1Blue, leave overnight at 37°C in a stirrer.
2. Dilute 1:100 the overnight culture in 400 ml of fresh LB.
3. Incubate at 37°C in a stirrer until OD₆₀₀ is about 0.3-0.4.
4. Spin 5 minute at 3000 rpm at 4°C.
5. Resuspend pellet in 40 ml of TFB (K-MES 10mM, pH 6.3; MnCl₂·4H₂O 45mM; CaCl₂·2H₂O 10mM; KCl 100mM and Hexaminecobalt chloride 3 mM).
6. Incubate 15 minutes on ice.
7. Repeat step 4, then resuspend in 16 ml of TFB.
8. Incubate at least for 1 hour on ice.
9. Add 240 μl of DMF (dimethyl formamide), keep 5 minutes on ice.
10. Add 240 μl of βMES (βMES 750mM, K-MES 10 mM, pH 6.3) and keep 10 minutes on ice.
11. Add 240 μl of DMF and keep 5 minutes on ice.
12. Add 560 μl of DMSO (dimethyl sulfoxide); mix gently and store on ice for 15 minutes.
13. Add an additional 560 μl of DMSO, mix gently and then return the suspension to an ice bath.
14. Dispense aliquots of the suspension into chilled microfuge tubes and immerse them in liquid nitrogen.
15. Store the tubes at -80°C until needed.

Heat Shock Protocol:

1. Thaw out an aliquot of XL1Blue cells from -80°C and keep on ice.
2. Add to these an aliquot of DNA or 15 μl of the ligation mixture.
3. Keep on ice almost for half an hour to favour contact between cells and foreign DNA.

4. Shock at 42°C in thermoblock for 2 minutes, then keep on ice for 2 minutes.
5. Centrifuge at 6000 rpm for 3 minutes.
6. Resuspend pellet in 500 µl of fresh LB, leave for 30 minutes in an agitation stirrer at 37°C.
7. Plate on selective solid LB plates; incubate overnight at 37°C.

3.7 Plasmid DNA purification

Once the desired colony has been identified through growth on selective medium, bacteria are inoculated in liquid selective medium (usually a rich medium like Luria-Bertani, with a suitable antibiotic) and incubated overnight at 37°C on a stirrer.

The purification type (miniprep, midiprep or maxiprep) depends on the desired amount of DNA: in optimum conditions and starting from 200 ml of liquid culture, maxiprep allows yields up to 1 mg of plasmid, so it is a procedure suitable to prepare a stock solution at high concentration (like 1 µg/µl).

Miniprep has a much lower yield since the starting culture volume is smaller: from 5 ml it is possible to recover 0.5-2 µg of plasmid, so it is useful for a rapid characterization of the clone through PCR or restriction enzyme analysis.

The following antibiotic concentrations have been used in this study to select the plasmid carrying bacteria: 100 µg/ml ampicillin, 50 µg/ml kanamycin, 25 µg/ml chloramphenicol.

Columns and solutions for this protocol are from “EuroGold Plasmid Miniprep kit” produced by Euroclone.

Miniprep protocol:

1. Transfer 1.5 ml of the overnight culture in a 1.5 ml test tube and centrifuge for 30 seconds at a maximum speed.
2. Pour off supernatant, if the bacterial pellet is too small add another 1.5 ml of overnight culture and repeat the centrifugation step.
3. Resuspend the bacterial cell pellet in 250 µl of Solution I (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml Rnase A stored at 4°C).
4. Add 250 µl of Solution II, lysis buffer (200 mM NaOH, 1% SDS), and mix by gentle inversion of the test tube. Wait 4 minutes at room temperature.
5. Add 350 µl of Solution III (potassium acetate 3 M pH 4.8). Gently invert the tube until a flocculent white precipitate forms.

6. Centrifuge the tube for 10 minutes at 14000 *rpm*.
7. The precipitate should have formed a pellet and the supernatant liquid should be clear. Transfer the clear supernatant to a fresh HiBind miniprep column in a 2 ml collection tube.
8. Centrifuge the tube for 1 minute at 12000 *rpm* to completely pass lysate through the membrane.
9. Discard the flow-through liquid and keep the column in the collection tube and add 750 μ l of Wash Buffer, completed with Ethanol absolute.
10. Centrifuge for 1 minute at 12000 *rpm*.
11. Discard the flow-through and repeat wash step with another 750 μ l Wash Buffer.
12. Repeat step 10.
13. Discard the flow-through and centrifuge again for 3 minutes at 12000 *rpm*. This step of drying is essential to remove any traces of ethanol from the column.
14. Place the column into a fresh 1.5 ml microcentrifuge tube.
15. Add 50-100 μ l of sterile water directly to the binding matrix and centrifuge for 1 minute at 6000 *rpm* to elute DNA.

Maxiprep protocol:

Columns and solutions for this protocol are from JETSTAR "Plasmid MAXI kit" produced by Genomed.

1. Transfer the overnight culture in a suitable centrifuge container and centrifuge at 2500 *rpm* for 45 minutes at 4°C.
2. Pour off the supernatant, remove all traces of medium carefully and resuspend the bacterial pellet in 10 ml of E1 solution (50mM Tris/HCl at pH 8, 10mM EDTA and RNase A 100 μ g/ml).
3. Transfer the suspension to a 50 ml test tube and add 10 ml of E2 lysis solution (200 mM NaOH, 1% SDS). Mix gently by inverting the test tube and wait 4 minutes.
4. Add 10 ml of E3 neutralization solution (3.1 M potassium acetate), mix immediately by multiple inverting until a homogeneous suspension is obtained.
5. Centrifuge at 10000 *rpm* for 10 minutes.
6. Load the supplied column with 30 ml of E4 equilibration solution (600 mM NaCl, 100 mM sodium acetate, 0.15% Triton X-100) and wait until the liquid has passed through the matrix.

7. Transfer the supernatant from step 5 to the column and wait until it has passed through the resin.
8. Load the column with 60 ml of E5 washing buffer (800 mM NaCl, 100 mM sodium acetate) and wait until the liquid has passed through the column.
9. Transfer the column on a new 50 ml test tube.
10. Load the column with 15 ml of E6 elution buffer (1500 mM NaCl, 100 mM Tris) and wait until the liquid has passed through the column.
11. Add 0.7 volumes of isopropanol (10.5 ml), mix thoroughly and centrifuge at 10000 *rpm* for 30 minutes at 4°C.
12. Remove the supernatant and wash the pellet with 0.4 ml of a 70% ethanol solution.
13. Transfer the suspension to a 1,5 ml test tube and centrifuge at 14000 *rpm* for 2-3 minutes.
14. Remove the supernatant, centrifuge again at the maximum speed for 30 seconds.
15. Remove any traces of ethanol, air dry and dissolve the pellet with filtered water.
16. Quantify by measuring 260 nm absorbance with a spectrophotometer. Dilute the solution to a suitable concentration; usually concentration of 1 µg/µl is desired.

3.8 Protein Extraction

Assorted detergents, salts, and buffers may be employed to encourage lysis of cells and to solubilize proteins. Protease and phosphatase inhibitors are often added to prevent the digestion of the sample by its own enzymes. Tissue preparation is often done at cold temperatures to avoid protein denaturing.

For Protein Extraction RIPA Buffer is required; it is composed of 250 mM NaCl, 50 mM Tris/HCl pH 7.4, 2mM EDTA and 1%NP-40; it should be kept at 4°C.

The full name of NP-40 is Tergitol-type NP-40, or nonylphenoxy polyethoxyethanol; NP-40 is a detergent not powerful enough to break the nuclear membrane, but can break the cytoplasmic membrane. As such, it can be used to obtain the cytoplasmic contents of a cellular culture.

When RIPA Buffer has to be used a protease inhibitor cocktail and PMSF (phenylmetansulfonide fluoride) should be added to the buffer, both 1: 100 of the final volume. PMSF and protease inhibitor should be kept at -20°C.

Protease inhibitor Cocktail (ALPHA Diagnostic International) set is a specially formulated cocktail of six protease inhibitors with broad specificity for the inhibition of aspartic, cysteine and serine proteases as well as aminopeptidases.

Is recommended for use with mammalian cells and tissue extracts.

This set contains AEBSF, Aprotinin, Bestatin, E-64, Leupeptin and Pepstatin, that are able to target serine proteases, aminopeptidase, cysteine protease, trypsin-like protease and aspartic proteases.

PMSF is an inhibitor of serine proteases and reacts with serine residues of trypsin, chymotrypsin, thrombin, papain and mammal acetylcholinesterase.

Protein Extraction protocol (for a 75 cm² flask):

Add to RIPA Buffer the protease inhibitor cocktail and PMSF, 1:100 of the final volume, and keep the buffer on ice until used.

1. Detach cells from the flask with a scraper.
2. Pellet cells at 3000 rpm for 10 minutes at 4 °C.
3. Discard the supernatant and resuspend pellet in 10 ml of cooled PBS 1X.
4. Pellet cells at 3000 rpm for 5 minutes at 4 °C.
5. Discard the supernatant and spin cells at 3000 rpm for 1-2 minutes, at 4 °C.
6. Discard all the supernatant with a pipette.
7. Resuspend pellet in 250 µl of RIPA buffer.
8. Incubate at least 30 minutes on ice.
9. Centrifuge at 14000 rpm for 15 minutes, at 4 °C.
10. Recover 200 µl of supernatant and add 50 µl of LAEMMLI 5x.
11. Denature at 56 °C for 15 minutes.
12. The extract so obtained can be used at the moment or stored at -20 °C until needed.

LAEMMLI 5X is composed as follows: 1.5g SodiumDodecylSulfate (SDS); 3,75 ml Tris/HCl, pH 6.8, 1M; 0.015 g Bromophenol Blue; 1.16 g DTT (dithiotreitol); 7.5 ml glycerol and H₂O to 15 ml.

The beta 2-mercaptoethanol reduces intra and inter-molecular disulfide bonds of the proteins to allow proper separation not by shape but by size. The amount of SDS bound is always proportional to the molecular weight of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide. SDS binds to all the proteins positive charges

which occur at a regular interval, thus giving each protein the same overall negative charge so that proteins will separate based on size and not by charge. The SDS also denatures the proteins and subunits to also help separate them based on size, and not on shape. SDS binds to proteins at about 1.4 g of SDS/g of polypeptide.

Bromophenol Blue serves as an indicator dye, and migration indicator where one can observe the dye front that runs ahead of the proteins. Bromophenol Blue also functions to make it easier to see the sample during loading of the gel well with protein sample. Glycerol in the Laemmli Buffer increases the density of the sample so that it will fall to the bottom of the well, minimizing puffing or loss of protein sample in the buffer, and layer in the sample well. DTT is present in many formulation to help reducing any disulphide S-S bonds that could provide secondary/tertiary structure and/or dimer formation.

3.9 Western blotting

The western blot (alternatively, protein immunoblot) is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies specific to the target protein. Samples may be taken from whole tissue or from cell culture. In most cases, solid tissues are first broken down mechanically using a blender (for larger sample volumes), a homogenizer (smaller volumes), or by sonication. Cells may also be broken open by one of the above mechanical methods. The proteins of the sample are separated using gel electrophoresis. Separation of proteins may occur by isoelectric point (pI), molecular weight, electric charge, or a combination of these factors. The nature of the separation depends on the treatment of the sample and the nature of the gel. This is a very useful way to determine a protein. The most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS), and it is used to separate molecules based on size, shape, or isoelectric point. SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents and heat to remove secondary and tertiary structure and to dissociate proteins and thus allows separation of proteins by their

molecular weight. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel.

Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size (usually measured in kilodaltons, kDa). The gel is a cross linked polymer matrix used to support and separate the molecules. The gel density can be controlled by varying the monomer concentration. Gels can be of constant density or they can be variable (gradient gels). The concentration of acrylamide determines the resolution of the gel - the greater the acrylamide concentration the better the resolution of lower molecular weight proteins. The lower the acrylamide concentration the better the resolution of higher molecular weight proteins. Samples are loaded into *wells* in the gel. One lane is usually reserved for a *marker* or *ladder*, a commercially available mixture of proteins having defined molecular weights, typically stained so as to form visible, coloured bands. When voltage is applied along the gel, proteins migrate into it at different speeds. These different rates of advancement (different electrophoretic mobilities) separate into *bands* within each *lane*.

SDS-PAGE is often carried out with a discontinuous buffer system in which the buffer used to cast the gel is different in ionic strength and pH respect to those used to run the gel. The SDS-polypeptide complexes in the sample are swept along by a moving boundary created when an electric current is passed between anode and cathode. Firstly samples migrate through a stacking gel of high porosity, then they are concentrated in a very thin stack on the surface of the resolving gel. The stacking gel contains Tris-HCl (pH 6.8), the buffer reservoir contains Tris-glycine (pH 8.3), and the resolving gel contains Tris-HCl (pH 8.8); all components of the system contain 0.1% SDS. The chloride ions in the sample and stacking gel form the leading edge of the moving boundary, and the trailing edge is composed of glycine molecules. Between the leading and trailing edges of the moving boundary is a zone of lower conductivity and steeper voltage gradient, which contributes to deposit the polypeptide on the surface of the resolving gel. The higher pH present in this zone favors the ionization of glycine, so glycinate migrates through the stacked polypeptide and travel through the resolving gel immediately behind the chloride ions. SDS-polypeptide complexes are then free to move through the resolving gel in a zone of uniform voltage and pH and can separate according to size by sieving.

Once proteins have been separated by SDS-PAGE, in order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of *nitrocellulose* or *polyvinylidene difluoride (PVDF)*. The membrane is placed on top

of the gel, and a stack of filter papers placed on top of that. The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it. Another method for transferring the proteins is called electroblotting and uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane. The proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection. Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Nitrocellulose membranes are cheaper than PVDF, but are far more fragile and do not stand up well to repeated problings. The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with Coomassie or Ponceau S dyes. Since the membrane has been chosen for its ability to bind protein, and both antibodies and the target are proteins, steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein (since the antibody is a protein itself). Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically Bovine serum albumin (BSA) or non-fat dry milk (both are inexpensive), with a minute percentage of detergent such as Tween 20. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces "noise" in the final product of the Western blot, leading to clearer results, and eliminates false positives.

During the detection process the membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme, which when exposed to an appropriate substrate drives a colourimetric reaction and produces a colour. For a variety of reasons, this traditionally takes place in a two-step process, although there are now one-step detection methods available for certain applications.

After blocking, a dilute solution of primary antibody is incubated with the membrane under gentle agitation. Typically, the solution is composed of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA. The antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight. It can also be incubated at different temperatures, with warmer temperatures being associated with more binding, both specific (to the target protein, the "signal") and non-specific ("noise"). After rinsing the membrane to remove unbound

primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. This is known as a secondary antibody, and due to its targeting properties, tends to be referred to as "anti-mouse," "anti-goat," etc.. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal.

In this case a horseradish peroxidase-linked secondary is used to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot.

Buffer required:

- Running Buffer 10X (250mM Tris, 206g glycine, 1%SDS, water to 1L)
- Transfer Buffer 10X (250 mM Tris, 144,1g Glycine and water to 1L)
- Transfer Buffer 1X (200 ml Transfer Buffer 10X, 400 ml Methanol, water to 2 L)
- TBST (100 ml TrisHCl 1M, pH 7.8, NaCl 1M, 0.5%Tween20, water to 1L)

Western Blotting Protocol:

1. Assemble the glass plates according to the manufacturer's instruction (Hoefer).
2. Prepare all the component of a 10 % resolving gel as follows: 4.8 ml of deionized water, 2.5 ml of Acrylamide Mix (Acrylamide-N,N'-methylene-bis-acrylamide, 29:1), 2.5 ml TrisHCl 1.5M, pH8.8; 100µl SDS (10% solution), 150µl Ammonium persulfate, APS, (10% solution in water, APS provides the free radicals that drive polymerization) and 6µl of TEMED (N,N,N',N'-tetramethylethylenediamine accelerates the polymerization by catalizing the formation of free radicals from APS). Mix the component and pour the solution as soon as possible into the gap generated between the glass plates. Deposits on the surface of the gel a thin layer on isobuthanol, to seal the gel.
3. After polymerization is complete, pour off the overlay and wash the top of the gel several times with deionized water; remove any traces of water with the edge of a paper towel.

4. Prepare the stacking gel as follows: 2.87 ml deionized water, 500µl of Acrylamide Mix, 500 µl of TrisHCl 1.0M, pH 6.8, 40 µl of SDS (10% solution),
5. 60 µl of APS and 6 µl of TEMED. Pour the gel through the spaces of the comb to completely fill in, being careful to avoid trapping air bubbles.
6. After polymerization is complete wash the wells several times with running buffer to remove any unpolymerized acrylamide, after mounting the gel in the electrophoresis apparatus.
7. Load up to 20 µl of each of the samples in to the bottom of the wells; attach the apparatus to an electric power supply and run until the bromophenol blue reaches the bottom of the resolving gel.
8. Remove the glass plates from the apparatus and prepare for electric transfer as follows: a sandwich of gel and PVDF membrane, between two pieces of towel paper and two sponges is compressed in a cassette and immersed in Transfer Buffer 1X in transfer apparatus. Constant Voltage is applied for 1 hour.
9. The membrane is incubated at least for 1 hour with a 5% skim milk solution in TBST 1X.
10. Skim milk is discharged and primary antibodies are added, antibody is diluted in 20 ml of a 1% Skim milk solution in TBST1X. The membrane has to be incubated in gentle agitation for 1 hour at room temperature or overnight at 4°C.
11. The blot is washed three times in TBST1X, and then secondary antibody is added to the membrane. After 1 hour incubation membrane is washed three times in TBST 1X, then detection is performed.
12. Place the membrane between two sheets of acetate. Gently lift the top sheet and with a pipette drop the chemiluminescent substrate (mix 1:1 the two solutions of luminal and peroxidase, from Millipore) on top of the membrane, scattering the drops over the surface of the membrane. Cover the membrane with the top sheet of plastic and remove all the bubbles present under the sheet, to create a liquid seal around the membrane.
13. Expose the membrane to an X-ray sheet for 3-5 minutes.

In this study were used one primary antibodies: Anti gD that reacts with an immunodominant gD (gIV) of 77 KDa of Bovine Herpesvirus 1 (from VMRD, Inc.). As a second antibody Anti mouse IgG (Whole molecule) peroxidase conjugated (from SIGMA) was used.

3.10 BAC recombineering

Recombineering (recombination-mediated genetic engineering) makes it possible to modify BAC DNA via homologous recombination, through the use of λ prophage recombineering system. Three λ Red-encoded genes (*exo*, *bet* and *gam*) are involved in the recombination system: *exo* encodes a 5'-3' exonuclease that produces 3' overhangs from introduced double strand foreign DNA; *bet* encodes a pairing protein that binds to this 3'overhangs and mediates annealing and homologous recombination with the complementary DNA on BAC and *gam* encodes an inhibitor of E.coli RecBCD exonuclease, thus protecting the linear DNA targeting cassette from degradation by RecBCD. These three genes are expressed under the control of the strong phage promoter pL; strictly controlled by the temperature-sensitive repressor, cI857; they are not expressed at 30°C, but are rapidly induced to very high level at 42°C for as little as 15 minutes.

Warming et al.¹²⁸ reported the development of a highly efficient *galk*-based positive/negative selection system for BACs's manipulation. The SW102 strain contains a complete galactose operon, except for a deletion in the *galk* gene. The *E.coli* galactose operon consists of four genes, necessary for growth and utilization of galactose as the unique carbon source. The *galk* gene product, galactokinase, catalyzes the first step in the degradation pathway of galactose, phosphorylating it to galactose-1-phosphate. It catalyzes also the efficient phosphorylation of 2-deoxy-galactose (DOG), a galactose analogue, to 2-deoxy-galactose-1-phosphate, a toxic compound, that can not be further metabolized. *Galk* selection can be used either for positive selection (using galactose as the only carbon source) than for negative selection (using DOG and glycerol as carbon source).

We developed a modified Recombineering system, based on *galk* selection and further modified by the introduction of a kanamycin resistance expression cassette, that drastically improved the background in the first targeting step (Donofrio et al., 2007). Here we report the detailed protocol for recombineering.

SW102 bacteria containing the galactokinase prokaryotic expression cassette, along with the kanamycin resistance expression cassette (KanaGalk) targeted into the Exon II of BoHV-4-U Immediate Early II (IE2) gene were grown, heat induced and electroporated with a gel purified fragment (-Left-IgK-E2-VP2²⁸⁰⁰-gD¹⁰⁶-Right-) obtained by cutting pORF50-IgK-E2-VP2²⁸⁰⁰-gD¹⁰⁶with PvuII (Fermentas). Here we report the complete

protocol for recombeneering.

Five hundreds μl of a 32°C overnight culture of SW102 bacteria containing BAC-BoHV-4-U Δ ORF50 (Franceschi et al., 2010), were diluted in 25 ml Luria–Bertani (LB) medium with or without chloramphenicol (SIGMA) selection (12.5 $\mu\text{g/ml}$) in a 50 ml baffled conical flask and grown at 32°C in a shaking water bath to an OD₆₀₀ of ~0.5- 0.6.

Then, 10 ml were transferred to another baffled 50 ml conical flask and heat-shocked at 42°C for exactly 15 min in a shaking water bath. The remaining culture was left at 32°C as the un-induced control.

After 15 min the two samples, induced and un-induced, were briefly cooled in ice/water bath slurry and then transferred to two 15 ml Falcon tubes and pelleted at 5000 *rpm* (Eppendorf centrifuge) at 0°C for 5 min.

The supernatant was poured off and the pellet was resuspended in 1 ml ice-cold ddH₂O by gently swirling the tubes in ice/water bath slurry. Subsequently, 9 ml ice-cold ddH₂O was added and the samples pelleted again.

This step was repeated once more, the supernatant was removed and the pellet (50 μl each) was kept on ice.

Electroporate cells with gel-purified ~4.428 kb fragment (-Left-IgK-E2-VP2²⁸⁰⁰-gD¹⁰⁶-Right-) obtained by cutting pORF50-IgK-E2-VP2²⁸⁰⁰-gD¹⁰⁶ with PvuII (Fermentas). An aliquot of 25 μl was used for each electroporation in a 0.1 cm cuvette at 25 μF , 2.5 kV and 201 Ω .

After electroporation, the bacteria were recovered in 10 ml LB in a 50 ml baffled conical flask and incubated for 4.5 h in a 32°C shaking water bath.

After the recovery period, the bacteria were washed twice in sterile 1x M9 salts (6 g/l Na₂HPO₄, 3 g/l KH₂PO₄, 1 g/l NH₄Cl , 0.5 g/l NaCl,) (SIGMA) as follows: 1 ml culture was pelleted in an eppendorf tube at 14.000 *rpm* for 15" and the supernatant was removed with a pipette. The pellet was resuspended in 1 ml of 1x M9 salts, and pelleted again.

This washing step was repeated once more. After the second wash, the supernatant was removed and the pellet was resuspended in 1 ml of 1x M9 salts. Washing in M9 salts is necessary to remove any rich media from the bacteria prior to selection on minimal medium plates.

Plate serial dilutions 100 μl each of 1:10, 1:100 and 1:1000 dilutions on M63 minimal medium plates containing 15 g/l agar (DIFCO, BD Biosciences), 0.2% glycerol (SIGMA), 1 mg/l d-biotin, 45 mg/l (SIGMA), l-leucine (SIGMA), 0.2% 2-deoxy-galactose (DOG,

SIGMA) and 25 µg/ml chloramphenicol (SIGMA).

Plates were incubated 3-5 days at 32°C. Several selected colonies were picked up, streaked on McConkey agar indicator plates (DIFCO, BD Biosciences) containing 20 µg/ml of chloramphenicol and incubated at 32 °C for 3 days until white colonies appeared. White colonies were grown in duplicate for 5–8 h in 1 ml of LB containing 50 µg/ml of kanamycin or LB containing 20 µg/ml of chloramphenicol. Only those colonies growing on chloramphenicol and not on kanamycin were kept and grown overnight in 5 ml of LB containing 20 µg/ml of chloramphenicol.

BAC-BoHV-4-A was purified and analyzed through BamHI restriction enzyme digestion for -Left-IgK-E2-VP2²⁸⁰⁰-gD¹⁰⁶-Right- fragment targeted integration.

Original detailed protocols for recombineering can also be found at the recombineering website (<http://recombineering.ncifcrf.gov>).

3.11 BAC DNA purification

Bacterial artificial Chromosomes (BACs) are synthetic vectors based on the fertility (F) factor of *E.coli* and are very attractive as a high capacity vector for genomic DNA because of their high stability and the ability of carry and propagate large insert (100-300 kb) of foreign DNA. Moreover a BAC is very easy to manipulate and because of its closed circular nature, it can be isolated from *E.coli* by classical alkaline lysis, even if, since BAC size is larger than commonly used plasmids, its purification requires more care in order to avoid shearing.

BAC extraction protocol (Warming et al., 2005):

1. In a 50 ml test tube, make a 5 ml overnight culture in LB with selected antibiotics at 30°C in agitation. The temperature is strictly restricted to 30-32°C.
2. Centrifuge at 3200 x g (7000 rpm) for 5 minutes.
3. Pour off the supernatant and put the test tube upside down on blotting paper in order to remove all traces of medium.
4. Resuspend the pellet in 250 µl of resuspension solution (50 mM Tris-HCl pH 8, 10 mM EDTA, 100 µg/ml RNase A) and transfer to a clean 1.5 ml test tube.

5. Add 250 μ l of lysis solution (200 mM NaOH, 1% SDS) and mix very gently by inverting the test tube. Incubate for no longer than 4 minutes at room temperature.
6. Add 250 μ l of sodium acetate, mix very gently and incubate on ice or at -80°C for 15 minutes.
7. Centrifuge at 14000 *rpm* for 5 minutes and transfer the supernatant to a clean 1.5 test tube.
8. Repeat step 7.
9. Add 750 μ l of isopropanol to precipitate DNA, mix gently and incubate on ice or at -80°C for 10 minutes.
10. Centrifuge at 14000 *rpm* for 10 minutes at 4°C.
11. Remove the supernatant by inverting the tube and wash the pellet with 0.5 ml of a 70% ethanol solution.
12. Remove all traces of ethanol and air dry the pellet.
13. Dissolve the pellet in 50 μ l of sterile filtered water.

3.12 Southern blotting

Southern blotting is a useful and powerful technique to identify and locate DNA sequences in large genomes.

First of all DNA which has to be analyzed must be broken up through restriction enzyme digestion to generate small fragments. These fragments will then be separated in an agarose gel by electrophoresis, denatured and transferred to a positively charged nylon membrane; UV treatment will result in immobilization of the fragment on the membrane.

A labelled probe is then used to search the fragments for the desired sequence: under stringent conditions the synthetic oligonucleotide will pair only to perfectly complementary sequences present on the membrane. Subsequent revealing of the probe tells whether the searched sequence was present or not in the analyzed genome.

Different labelling methods are used: the first one employed involves radioisotopes; revealing of DNA duplex is obtained through autoradiography.

For this study a non radioactive labelling methods was used: the sequence of interest has been amplified through PCR, but in the reaction mixture digoxigenin labelled dUTP was added. The result is an oligonucleotide with several thymines replaced by this modified nucleotide: revealing of the probe is carried out by a specific antibody conjugated with alkaline phosphatase. Giving the adequate substrate (CSPD from Roche), the

phosphatase converts it to a luminescent molecule: subsequent exposure of an X-ray sheet to the membrane results in revealing of DNA duplex.

In this study purified BAC clones from *E.coli* have been digested for 6 hours with an appropriate restriction enzyme, then the fragments have been separated overnight through agarose gel electrophoresis with a voltage of 25 V and transferred (according to the protocol) to a nylon membrane (by Boheringer Mannheim).

Here a protocol to produce labelled probes is presented along with a southern blotting protocol (Sambrook and Russell, 2001).

Probe labeling reaction:

The size of the probe should be between 500 and 1000 bp; labelling PCR reaction should be made as the following one:

- 5 µl of dNTPs mix 10X (2mM)
- 5 µl of *Taq* buffer 10X (from Invitrogen)
- 5 µl of *Taq* buffer 10X (from Invitrogen)
- 5 µl of primers 10X (2.5 µM each)
- 1 µl of template (1 µg of plasmid or preamplified fragment)
- 1 µl of Digoxigenin-11-dUTP alkaline labile (Roche)
- 1 µl of *Taq* polimerase 5U/ µl (Invitrogen)
- H₂O to 50 µl

Every cycle comprises 1 minute at 94°C, 1 minute at 55°C, a minute at 72°C; the number of cycles is 35.

Because incorporation of digoxigenin-dUTP results in a higher molecular weight than non labelled oligonucleotides, a parallel reaction without this modified nucleotide should be performed to check incorporation of digoxigenin-dUTP: the labelled amplicon should migrate less than the control in an agarose gel electrophoresis.

Southern blotting protocol:

1. After separation of DNA fragments by electrophoresis, put the agarose gel (bottom up) in a container with 250 ml of depurination solution (0.25 M HCl) in a shaking waterbath. This solution will remove some purines from DNA, preserving the deoxyribose-phosphate backbone, in order to facilitate transfer of the DNA to the membrane. Incubate for 15 minutes or until the dye from the loading buffer changes colour from blue to yellow.
2. Remove depurination solution and add 250 ml of denaturation solution (1.5 M NaCl, 0.5 M NaOH). Incubate for 15 minutes in a shaking waterbath or until the dye changes back its colour to blue.
3. Remove denaturation solution and add 250 ml of neutralization solution: 1.5 M NaCl, 0.5 M Tris pH 7.5, 1mM EDTA. Incubate for at least 20 minutes.
4. Bring agarose gel into contact with the nylon membrane as shown in figure:

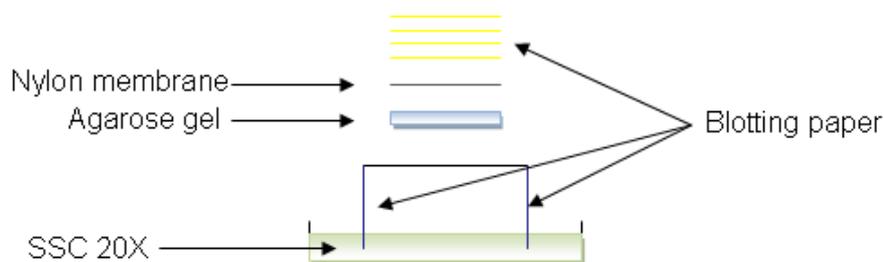


Figure 3.1 General diagram of a DNA transfer from an agarose gel to a nylon membrane

SSC 20X has the following composition: 3 M NaCl, 0.3 M sodium citrate. Leave overnight: SSC buffer will be drained from the lower reservoir by capillarity and it will transfer DNA from the gel to the membrane.

5. Transfer the membrane to an ultraviolet oven and crosslink DNA in order to covalently bind it to the membrane.
6. Put the membrane in a glass tube and add 50 ml of pre-hybridization solution (7% SDS, 0.5 M Na₂HPO₄). Incubate at 65°C in a rotisserie for half an hours.
7. Put 5 µl of PCR labelling reaction and 500 µl of sterile filtered water in a 2 ml screw cap test tube. Put the test tube in boiling water for 5 minutes to denature DNA, then put it immediately on ice.
8. Transfer the probe to 50 ml of hybridization solution (7% SDS, 0.5 M Na₂HPO₄, 1mM EDTA).

9. Replace the pre-hybridization solution in the glass tube with the hybridization solution. Incubate overnight at 65°C in a rotisserie (Tecna instrument oven).
10. Pour off hybridization solution.
11. Wash the membrane with 100 ml of washing solution 1 (0.5X SSC, 0.1% SDS) for 1 hour at 65°C.
12. Pour off washing solution 1 and repeat step 11.
13. Remove washing solution 1 and add 100 ml of washing solution 2 (40 mM PO₄ pH 7.2, 0.05% SDS). Incubate for 30 minutes at 65°C.
14. Repeat step 13.
15. Pour off washing solution 2 and equilibrate the membrane with washing solution 3 (100 mM Maleic acid, 150 mM NaCl, 0.3% Tween) for 1 minute at room temperature in a shaker.
16. Remove washing solution 3 and block the membrane by gentle agitation for 30-60 minutes in blocking solution (100 mM Maleic acid, 150 mM NaCl and 1% of blocking reagent from Roche).
17. Dilute the Anti-Digoxigenin-AP (Fab fragment 150U/200 µl, Roche) 1/15000 in 50 ml of blocking solution. Pour off the blocking solution and incubate the membrane for 30 minutes in the antibody solution.
18. Discard the antibody solution. Gently wash the membrane twice, 15 minutes per wash, with 100 ml of washing buffer 3.
19. Pour off washing buffer 3 and equilibrate the membrane in detection buffer (100 mM Tris/HCl, 1 mM EDTA pH 9.5) for two minutes.
20. Place the membrane between two sheets of acetate. Gently lift the top sheet and with a pipette drop the chemiluminescent substrate (CSPD from Roche) on top of the membrane, scattering the drops over the surface of the membrane. Cover the membrane with the top sheet of plastic and remove all the bubbles present under the sheet, to create a liquid seal around the membrane. Leave the membrane at room temperature for 20 minutes.
21. Expose the membrane to an X-ray sheet for 3-5 minutes.
22. Develop the sheet.

In this study a probe for gE2 was generated by PCR using this primers:

- BVDVsecSense: 5'-ccc gaa gct tgc act tgg att gca aac ctg aat tc-3'
- BVDVsecAntisense: 5'-ccc cgc tcg aga gtg gac tca gcg aag taa tcc cg-3'

4 RESULTS AND DISCUSSIONS

4.1 BTV-8 VP2 cDNA from pBRT7VP2

The pBRT7VP₂ plasmid vector contains the full length of BTV-8 VP₂ ORF cDNA, 2886 bp. The complete sequence without ATG and Stop codon was amplified with the primer pair: Blue-sense with *HindIII* site and Blue-antisense with *Sall* site.

a)



b)

```

atggaggactagcattccgatttatacgaatgtattcccagcggagcttttagatgga
M E E L A I P I Y T N V F P A E L L D G
tatgattacatcattgatgtagtagtgcgtggtgaaggaaggtgatgagcccgttaag
Y D Y I I D V S S R V E E E G D E P V K
cgacacgagttaacagagattcctaggaattcaatgttcgatattaaggatgaacatata
R H D V T E I P R N S M F D I K D E H I
cgtgatgcgattatacaaacacgctcaacaatgatgggtacgtactaccgagagattata
R D A I I Y K P V N N D G Y V L P R V L
gatatacagctgaaagcctttgacgaccgaaagaggtgtttgaaatgatggtcatagc
D I T L K A F D D R K R V V L N D G H S
gaatttcatacgaaaaacgaattgggttcagtggtgatgatgacgcaatggtatccaa
E F H T K T N W V Q W M I D D A M D V Q
cctcctaaggtagatgcctcacacgcgctcgaggatcagccacgcaactccttaactgt
P L K V D I A H T R S R I S H A L F N C
acggtgcgattgcattcaaaaagctgacacgcgcatcttatcagttgaaccagttgaa
T V R L H S K K A D T A S Y H V E P V E
attgaatcgtggggtgtaaccacacatggttgatgaggtatcatcatctagtgaaatgtt
I E S W G C N H T W L S R I H H L V N V
gaattattcattggttcaacaagggcgggtatatacattgaaacccacatataagataata
E L F H C S Q E A A Y T L K P T Y K I I
tcaaatgcggaacgctgcatcaacgagtgattcctttaaaggactatgatcgaattaggt
S N A E R A S T S D S F N G T M I E L G
cgtaatcatcaaatcagatgggtgaccagggataccagaagttgaaagaaggggtggtg
R N H Q I Q M G D Q G Y Q K L K E G L V
caagttcgaattgaaaggaagacgcttggtaatacaagaggaataaacgcttgaat
Q V R I E G K T P L V I Q E E I T A L N
aaaataagagaacaatggatcgctcgaatttcgatcaaaaggaatacaaggttttagat
K I R E Q W I A R N F D Q R E I K V L D
ctgtgtaggttgttctacgataggttaggaagatgtgcaactactgaagaggaacctaag
L C R L L S T I G R K M C N T E E E P K
aatgaagctgatcttccagtgaaattccaatggagcttgacgaaatatttcgacggga
N E A D L S V K F Q M E L D E I F R P G
aataacgagcgtaccaacatcatggggggcggagatcatcggaacacgaggaagattt
N N E R T N I M G G G V H R K N E D R F
tactgactaattatgatcgctgcatccgataccaataaaggtcgtatattggtggacaat
Y V L I M I A A S D T N K G R I W W S N
ccgatatcctgctcgctggagcgttaatcgccgctgaagccaattgggtgatgtttac
P Y P C L R G A L I A A E V Q L G D V Y
aatcctttaaaggaattgggtccaatggagcgtacgacactacatgatccttacgatagg
N L L R N W F Q W S V R P T Y V P Y D R
aatagagaaggtgataagatatttatagtagaatacaattatttgattcaacctgaga
N R E S D K Y I Y S R I N L F D S T L R
ccagcgcaaaaatagatcattgggagatcaaggtccttaaggaagtagcagaggttagc
P G D K I V H E Y K L L N E V R E V S
atcaacaaggtatgagtcgcatctgctcctgaggtgaagaattaccaccaagttt
I N K G N E C D L F P E D E E F T T K F
catgagcgcggtatcaggagatgaagaacaaaattcaaaagcgggtggaatcaacga
H E A R Y T E M K N Q I I Q S G W N Q R
gattttaaataactgataaataactagaggtggcgcaaatgtgtaacgattgatttag
D F K M H K I L E D G A N V L T I D F E

```

```

aaggacgcacatcggaacgggttcggcctgagtttgcggtactatacaaaatgg
K D A H I G T G S A L S L P D Y Y N K W
ataatgtccgattggttaacgctaaagttacgcattaccgaggttggattggaacggct
I I A P M F N A K L R I T E V V I G T A
cacaccgagatccagctgtaggcgagcgcgaagcgttcacacatgatccttttgac
H T D D P A V G R S A K A F T H D P F D
ctgcagcgtatgtctagcaagatactatgatgtacgacctggtatgatgggcccgcgt
L Q R Y C L A R Y Y D V R P G M M G R A
ctctctaagcaaaaaatgtcgtcgtgactgataaactctctaagcagagagattac
L S K Q Q N M S S M T D K L S K Q E D Y
gcagggattgttcaaggcgtctggaatacaaggaacgagagaacagatgcttgacggag
A G I V S R R L E Y K E R E N R C L T E
acagcgcagatgtattcgagaaaacatgcttatacgtactagaattatgagcagacat
T A Q Y V F E K T C L Y V L E L L S R H
acgatgccctcggaggtctgaagtacgttgaacacccaacaattgatcccagcgtc
T M P S E D S E V T F E H P T I D P S V
gatattgaaacgtgaaataaattgatggttgcgaactgatcatattcgtattgactat
D I E T W K I I D V S Q L I I F V F D Y
ttggtgaaatcggaagatcgtgagggatactaccgaggaaggtggacgctttcaaa
L F E N R K I V R D T T E A R W T L F K
ataagatcggaagtaggacgagccaggttgacgcaatagagatgactttcctcgggtt
I R S E V G R A R I D A I E M T F P R F
ggacgtatgctaagaatgctctcaggcaagaataaatacaagatcatcgctgctgaa
G R M L R N A S Q A K I N Q D I A C L N
ttccttccactctttcatcatagggcgaataatcttacgcacatagacagtgaggt
F L P L L F I I G D N I S Y A H R Q W S
ataccggtcgtcgtgatgagcagatataccaatataccttggaaagtgagcgtac
I P V L L Y A H D I R I I P L E V G A Y
aataacgcttgggctgacgtcaactacagatataatggcttcttccatcgatagct
V K G I A L I S V D R N Q K V S V Q T E
gnnrfgltsyleymaffpsya
actagtagtctaaaatgacgagtcgatcaaggagtcgagcagcgaatggctgagttt
T R V A K I D E S I K E C A I A M A E F
tatatgaacaccgatatacagctggagtgtagtagtaacgtgataacgacgaagcgg
Y M N T D I H S G S V M S N V I T T K R
ctatgtatgaacatacttagctcgtatcggcgggttattcggatggattgctttwg
L L Y E T Y L A S L C G Y S D G L L W
tacctgcaactacacatcctcgaatgcttagtcgcttgcggttaggtgatgacgta
Y L P I T H P S K C L V A F E V A D D V
gttccaactcagtaaggcgggaacgtattctctatctaggttcccttaagctcgagacat
V P L S V R R E R I L S R F P L S S R H
gtcaaggggattgcgttattcgggtgagtaggaatcaaaaggtatcggttcaaacagag
V K G I A L I S V D R N Q K V S V Q T E
ggtattgtaacacatcgtctcgtgaaaagaatcgtgaaaacagctggtgatgttatt
G I V T H R L C K K N L L K Y V C D V I
cttttaagtttctggacagttttgggaatgatgagatgtaactaagctgctcaat
L F K F S G H V F G N D E M L T K L L N
gtatag
V -

```

c)

Primer name	Sequence
Blu-sense	5'-ccccga▼ <u>agc ttg</u> gag gag cta gcg att ccg att tat acg aat-3' HindIII
Blu-anti	3'-cta ctc tac aat tga ttc gac gag tta cat <u>cag ct▲</u> gagcccc-5' Sall

Figura 4.1 a) Diagram (not to scale) shows the pBRT7VP2 plasmid vector. b) Top line shows VP2 ORF sequence, lower line shows translated sequence. In red the ATG and Stop Codon. c) The sequence of the primers Blue-sense with *HindIII* site and Blue-antisense with *Sall* site.

4.2. plgkVP2 generation

plgKVP₂ is the first VP₂ expression vector, in which VP₂ ORF lacking in ATG and Stop Codon is sub-cloned in frame following the immunoglobulin kappa light chain (Igk) signal peptide, specifying secretion of heterologous proteins.

In order to achieve this first construct, VP₂ amplicon, amplified with Blu-sense and Blu-antisense primer pair, is sub-cloned in pSecTag2/Hygro A (Invitrogen, Milano, Italy) in frame with the IgK signal peptide, after HindIII/SalI double digestion; in this way plgKVP₂BGH Intermediary Construct has been obtained. After that, NheI/IgKVP₂/SalI fragment from plgKVP₂BGH Intermediary Construct was sub-cloned in pEGFP-C1 (Clontech) lacking in HindIII/EGFP sequence/SalI fragment.

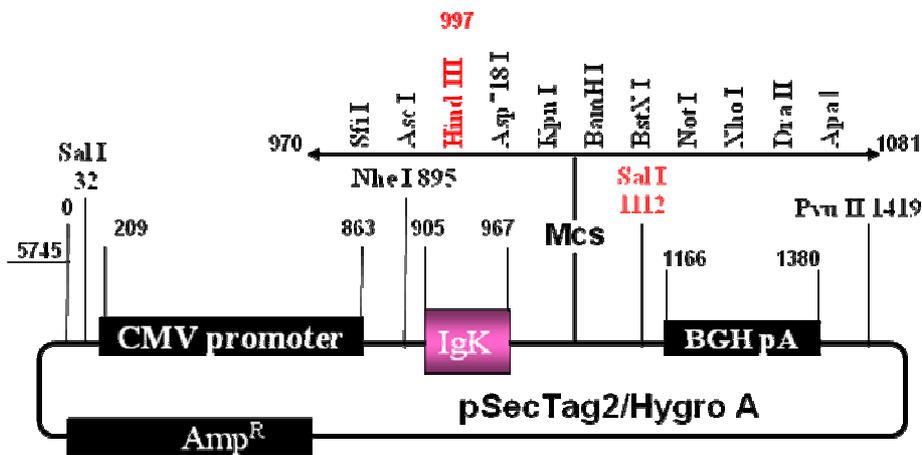


Figure 4.2 Diagram (not to scale) shows the organization of pSecTag2Hygro A plasmid. Particularly, Human cytomegalovirus immediate-early enhancer promoter, bases 209-863 (CMV, black); IgK signal peptide, bases 905-967 (IgK, pink); the Mcs, Multiple cloning site, bases 970-1081; the BGH (Bovine growth Hormone) polyadenylation signal, bases 1166-1380 and the gene to ampicillina resistance, AmpR, bases 4749-5609.

STEP 1 : in pSecTag2/Hygro A was sub-cloned HindIII/VP₂ Amplicon/SalI fragment.

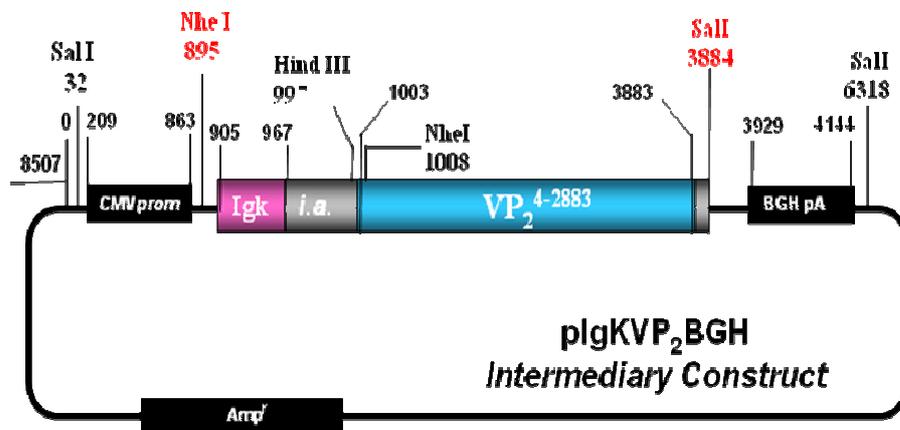


Figure 4.3 Diagram (not to scale) shows the construct plgKVP₂BGH Intermediary Construct containing: Human cytomegalovirus immediate-early enhancer promoter bases 209-863 (CMV, black), IgK signal peptide bases 905-967 (IgK, pink) in frame with VP₂ full ORF lacking in ATG and STOP codon (VP₂, blue); two intergenic areas are shown in gray: the first is brought by pSecTag2Hygro vector (the sequence between plgK and HindIII site) and Blu-sense primer; the second is brought by Blu-antisense primer.

STEP 2: NheI/IgKVP₂ /Sall fragment from plgKVP₂BGH Intermediary Construct was subcloned in pEGFP-C1 cut with NheI and Sall

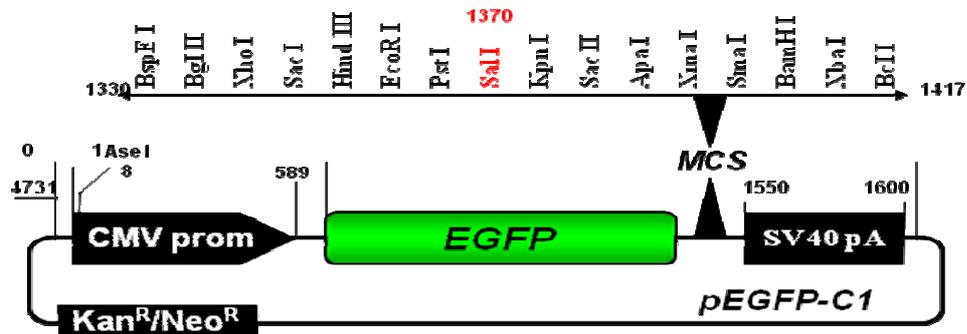


Figure 4.4. Diagram (not to scale) of pEGFP-C1 plasmid . Particularly: CMV promoter (base 1-589) in black, EGFP (bases 606-1410) in green, Mcs (multi cloning site, bases 1330-1417) and the SV40 polyadenylation signal (bases 1550-1584) in black.

Thus, the first VP₂ expression vector, plgKVP₂, was obtained.

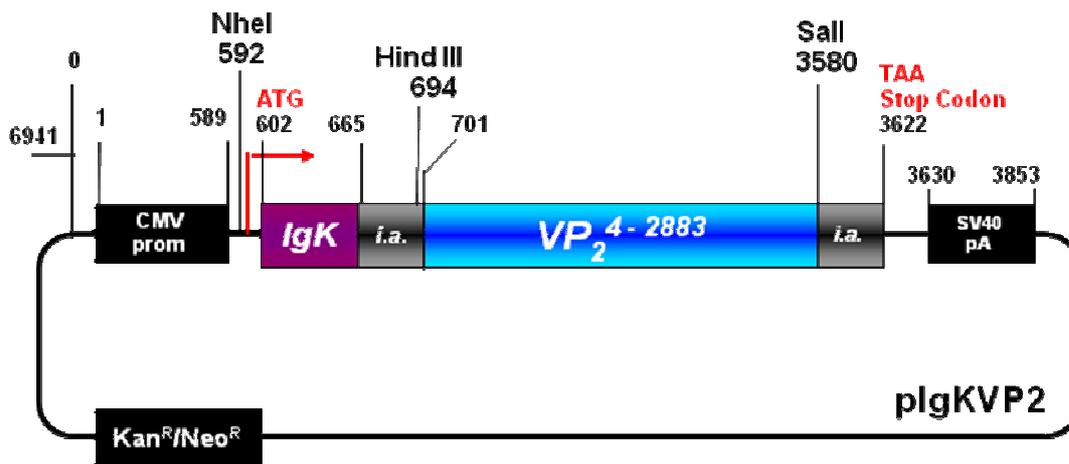


Figure 4.5. Diagram (not to scale) shows plgKVP2 construct. Particularly: cytomegalovirus enhancer promoter (CMV) in black; IgK signal peptide (IgK) in magenta and in frame with the VP₂⁴⁻²⁸⁸³ ORF in blue; between them there is the first intergenic area (i.a.) and downstream the VP₂⁴⁻²⁸⁸³ ORF there is a second intergenic area (in grey) brought by pEGFP-C1 backbone until the TAA Stop codon. SV40 polyadenylation site (SV40 pA) in black and the kanamycin/neomycin resistance gene in black.

4.3 VP2 Tag generation

Due to the lack of a suitable monoclonal antibody against BTV-8 VP2, it was not possible to use plgKVP2 to assess the expression and secretion of VP2 in mammalian cells through the Western blotting. So we used plgKVP2 as a backbone in which we inserted a suitable tag. Firstly, we used a commercial marked system like His6, CMyc and HA, but we had a strong background, so it was impossible to distinguish between VP2 specific band and others. Furthermore a tag soluble epitope peptide belonging to Bovine herpesvirus 1 (BoHV-1) glycoprotein D (gD) ectodomain was mapped using a commercial corresponding Anti-gD monoclonal antibody (1B8-F11, VMRD, Pullman, USA). Several fragments of the soluble ectodomain gD ORF were cloned in frame at the 3' end of VP2 ORF into plgKVP2 to get **plgK-VP2-gD₃₆₁**, **plgK-VP2-gD₁₈₁**, **plgK-VP2-gD₁₂₇**, **plgK-VP2-gD₁₀₆** and **plgK-VP2-gD₉₃**.

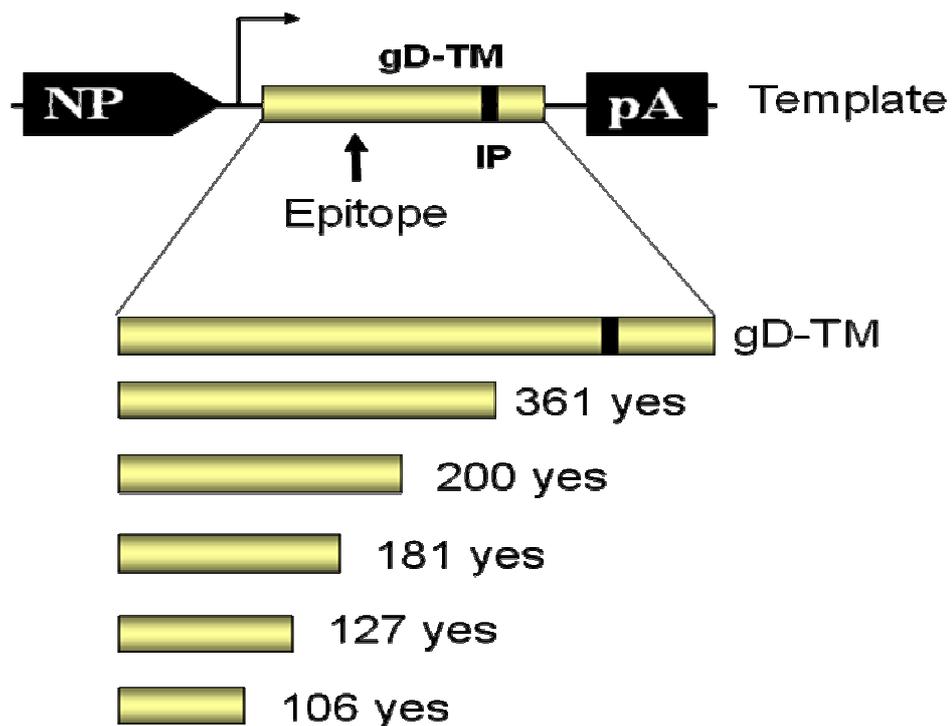


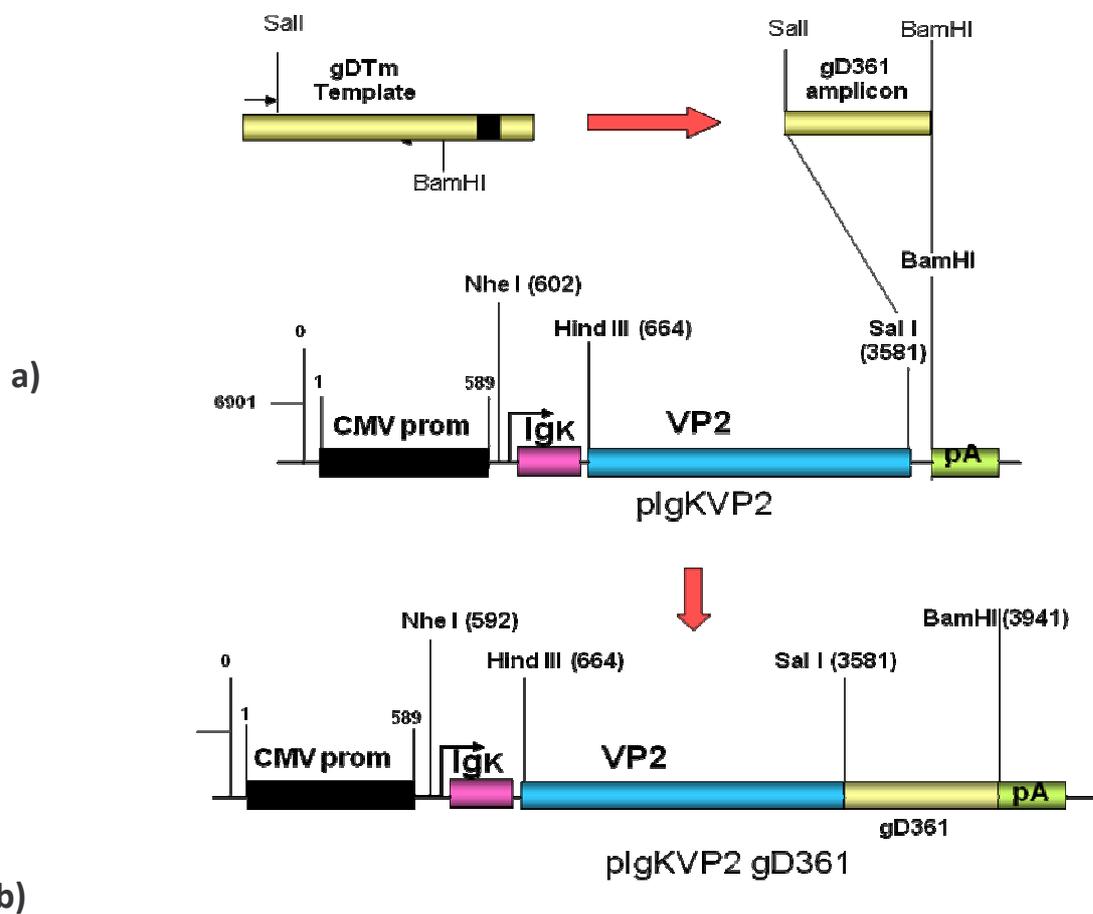
Figure 4.6. Diagram (not to scale) showing the expression cassettes included in the vectors employed throughout the study: the pgD vector, containing the gD natural promoter (NP) and the gD ORF with the BGH polyadenylation signal (pA). mAb epitope **mapping**

pIgK-VP2-gD361

Strategy :

A fragment of gD was generated by PCR from pgD¹²⁹ using gDtag-sense-361 and gDtag-anti-361. *Sall* site is contained in the 5' end of the amplicon just following sense primers, rather, *BamHI* site is contained into the 3' end of the antisense primer.

So generated amplicon was cut with *Sall* and *BamHI* and the resulting 361 fragment sub cloned into plgKVP2 which was cutting with *Sall* and *BamHI* (VP2 is in frame with gD fragment).



Primer name	Sequence
gDtag-sense-361	5'-ccg cag gag gca cac aag gcc ata g▼tc gac tac tgg ttc -3' SalI
gDtag-anti-361	3'-cgg ggg cga tgc ggg cgc cgg ggg ctg cgg att cct ag▲ggcgatt -5' BamHI

Figure 4.7. a) The strategy to obtain plgKVP2gD361 and construct, containing: cytomegalovirus enhancer promoter (CMV, black), IgK signal peptide (IgK, pink) in frame with VP2 full ORF (VP2, blue) and gD361 (gD361, yellow). **b)** the sequence of the primers.

The obtained construct was electroporated into HEK 293 T cells (as previously described)¹³⁰.

Cells and culture medium were checked by western immuno-blotting using anti-gD antibody for VP2 expression analysis.

Expression was tested into cell fraction and sovranatant of transiently transfected HEK 293T cells by western blotting.

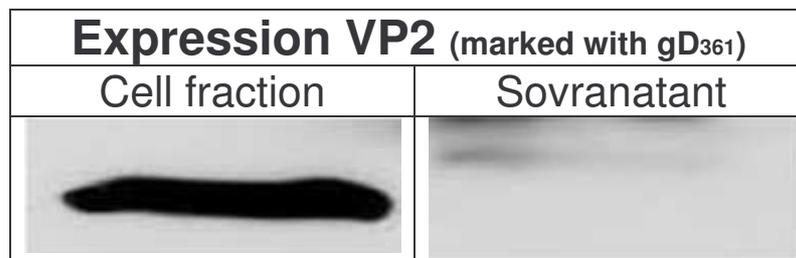


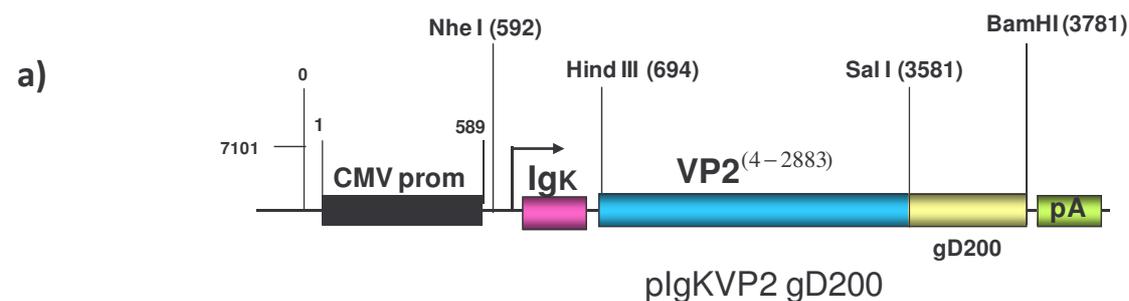
Figure 4.8 The VP2 expression (marked with gD₃₆₁) in Mammalian cells. It presents the extracts and the sovranatant.

pIgK-VP2-gD200

Strategy:

gD fragment was generated by PCR with the primers gD-Tag-sense and gD-Tag-anti-200. Sall site is contained in the 5' End of the amplicon just following the sense primers, rather Bam HI site is contained into the 3' end of the antisense primer.

So generated amplicon was cut with Sall and BamHI and the resulting 200 fragment sub cloned into pIgKVP2 cutted with Sall and BamHI (VP2 is in frame with gD fragment).



b)

Primer name	Sequence
gDtag-sense	5'-ccg cag gag gca cac aag gcc ata g▼tc gac tac tgg ttc -3' Sall
gDtag-anti-200	3'-ccg ccg ggg ggt cct ggg ctt ccg ctg ccg att cct ag▲ggcgatt -5' BamHI

Figure 4.9 a) The construct containing: cytomegalovirus enhancer promoter (CMV, black), the IgK signal peptide (IgK, pink) in frame with VP2 full ORF (VP2, blue) and gD200 (gD200, yellow). **b)** the sequences of the primers.

So derived construct was electroporated into HEK 293 T cells (as previously described)¹³⁰ and cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and surnatant of transiently transfected HEK 293T cells by western blotting.

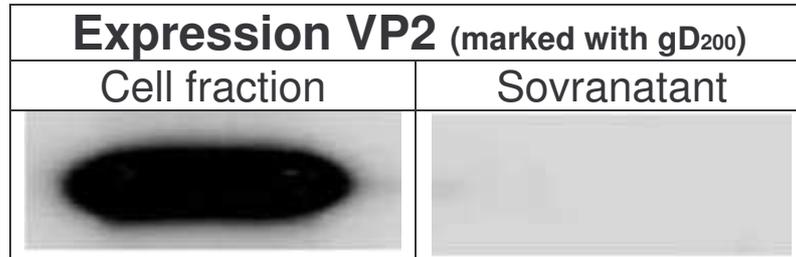


Figure 4.10. The VP2 espression (marked with gD200) in Mammalian cells and the presence in cell extract and in sovranatant.

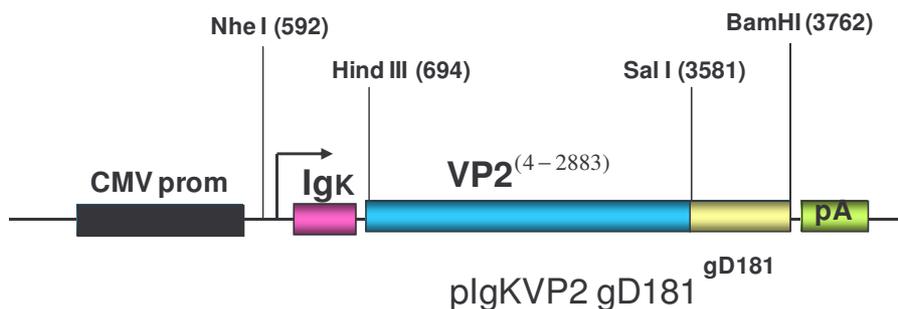
plgK-VP2-gD181

Strategy:

A fragment of 181 bp was generated by PCR from pgD using primers pairs containing *Sall* site restriction in the 5' end of the amplicon just following sense primers, rather, Bam HI restriction site is contained into the 3' end of the antisense primer.

So generated amplicon was cut with *Sall* and *BamHI* and the resulting 181 fragment sub cloned into *plgKVP2* by cutting with *Sall* and *BamHI* (*VP2* is in frame with *gD* fragment).

a)



b)

Primer name	Sequence
gDtag-sense	5'-ccg cag gag gca cac aag gcc ata g▼tc gac tac tgg ttc -3' Sall
gDtag-anti-181	3'-cgt cgg ccc gcc ctc ccg ttg att cct ag▲ggcgatt -5' BamHI

Figure 4.11 a) showing the construct containing: The cytomegalovirus enhancer promoter (CMV, black), the IgK signal peptide (IgK, pink) in frame with VP2 full ORF (VP2, blue) and gD181 (gD181, yellow). **b)** are showed the sequence of the primers.

Cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and sovranatant of transiently transfected HEK 293T cells by western blotting.

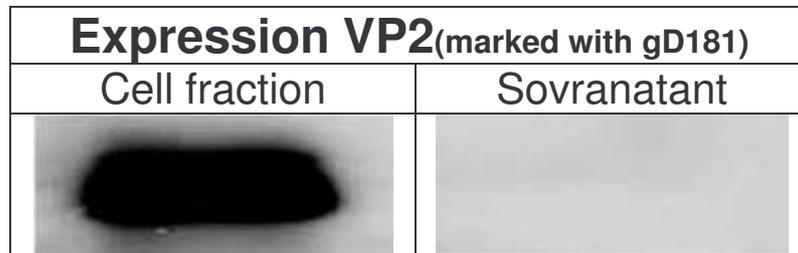
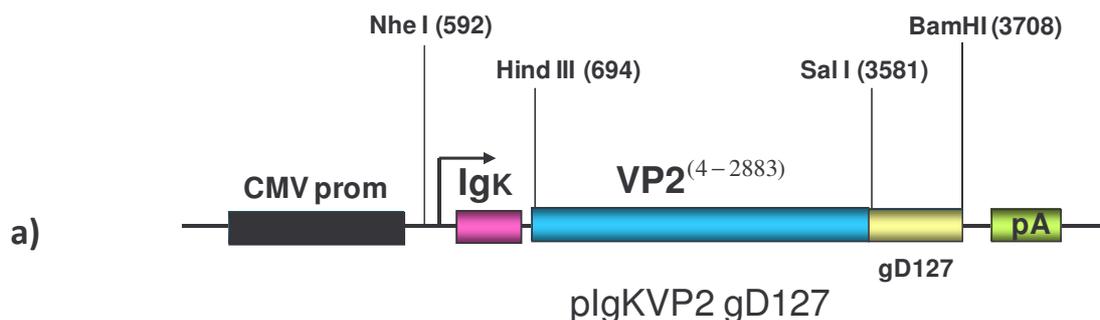


Figure 4.12. The VP2 expression in Mammalian cells and the present in cell extract and in sovranatant.

pIgK-VP2-gD127

Strategy:

- 1) A gD fragment was generated by PCR with the primers gD-Tag-sense and gD-Tag-anti-127. *Sall* site is contained in the 5' end of the amplicon just following sense primers, rather, *Bam HI* site is contained into the 3' end of the antisense primer.
- 2) So generated amplicon was cut with *Sall* and *BamHI* and the resulting 127 fragment sub cloned into plgKVP2 by cutting with *Sall* and *BamHI* (VP2 is in frame with gD fragment).



b)

Primer name	Sequence
gDTag-sense	5'-ccg cag gag gca cac aag gcc ata g▼tc gac tac tgg ttc -3' SalI
gDTag-antisense127	3'-cgc ggt ggg ccg ctg ctg ctc att cct ag▲ggcgatt -5' BamHI

Figure 4.13. a) are showed strategy to obtained pIgKVP2gD127 and showing the construct containing: The cytomegalovirus enhancer promoter (CMV, black), the IgK signal peptide (IgK, pink) in frame with VP2 full ORF (VP2, blue) and gD127 (gD127, yellow). **b)** are showed the sequence of the primers.

This construct was electroporated into HEK 293 T cells (as previously described)¹³⁰, cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and sovranatant of transiently transfected HEK 293T cells by western blotting.

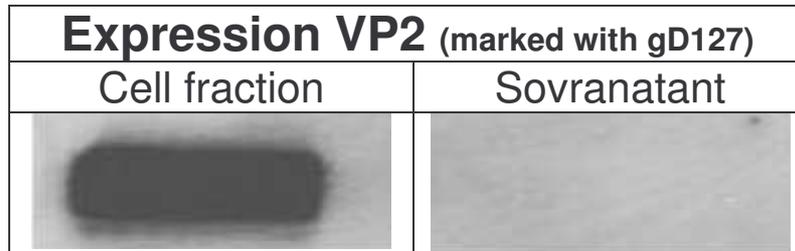
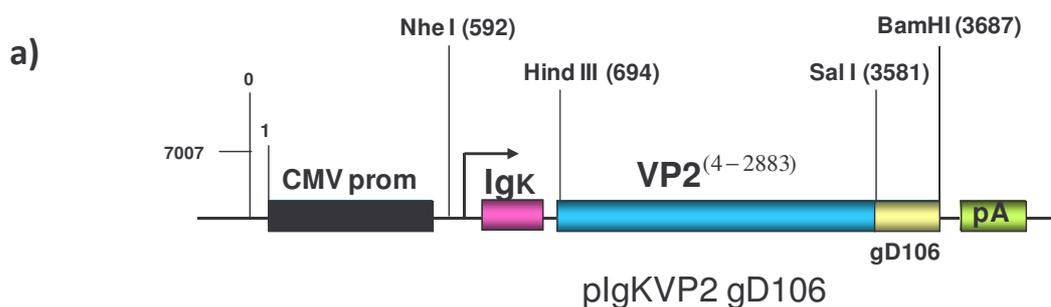


Figure 4.14. The VP2 expression (marked with gD127) in Mammalian cells and the present in cell extract and in sovranatant.

pIgK-VP2-gD106

Strategy:

1. gD₁₀₆ fragment was generated by PCR with the primers gD-Tag-sense and gD-Tag-anti-106. *Sall* site is contained in the 5' end of the amplicon just following the sense primers, rather, *BamHI* site is contained into the 3' end of the antisense primer.
2. So generated amplicon was cut with *Sall* and *BamHI* and the resulting 106 fragment sub cloned into plgKVP2 cutted with *Sall* and *BamHI* (VP2 is in frame with gD fragment).



b)

Primer name	Sequence
gDTag-sense	5'-ccg cag gag gca cac aag gcc ata g▼tc gac tac tgg ttc -3' SalI
gDTag-antisense106	3'-cgg cgg cta ccc cca agg ggg att cct ag▲ggcgatt -5' BamHI

Figure 4.15. a) The plgKVP2gD₁₀₆ construct containing: cytomegalovirus enhancer promoter (CMV, black), IgK signal peptide (IgK, pink) in frame with VP2 full ORF (VP2, blue) and gD₁₀₆ (gD₁₀₆, yellow). **b)** the sequence of the primers

The obtained construct was electroporated into HEK 293 T cells as previously described 130.

Cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and surnatant of transiently transfected HEK 293T cells by western blotting.

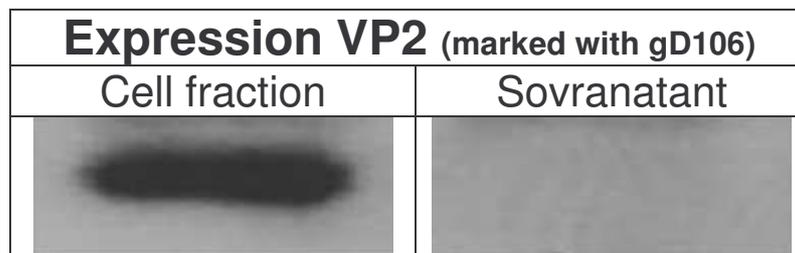
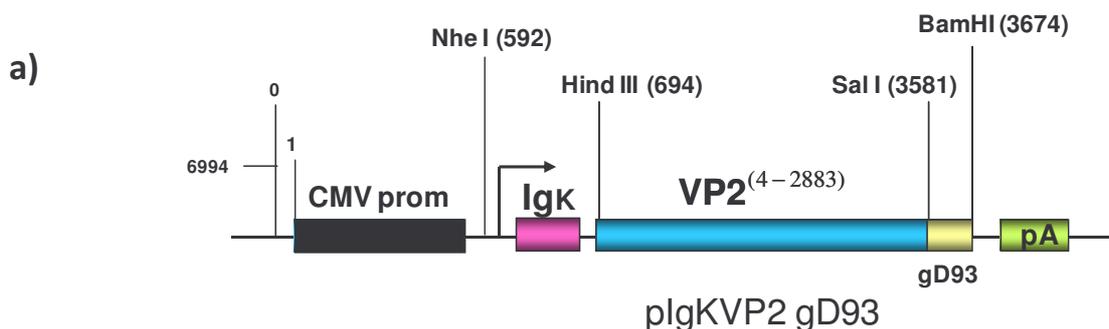


Figure 4.16. The VP2 expression (marked with gD106) in Mammalian cells and the present in cell extract and in sovranatant

pIgK-VP2-gD93

Strategy:

1. gD93 fragment was generated by PCR with the primers gD-Tag-sense and gD-Tag-anti-93. *Sall* site is contained in the 5' end of the amplicon just following the sense primers, rather, *BamHI* site is contained into the 3' end of the antisense primer.
2. So generated amplicon was cut with *Sall* and *BamHI* and the resulting 93 fragment sub cloned into plgKVP2 by cutted with *Sall* and *BamHI* (VP2 is in frame with gD fragment).



b)

Primer name	Sequence
gDTag-sense	5'-ccg cag gag gca cac aag gcc ata g▼tc gac tac tgg ttc -3' SalI
gDTag-antisense93	3'-ctc ggc ggc gga cgg cgg cta att cct ag▲ggcgatt -5' BamHI

Figure 4.17. a) The plgKVP2gD93 construct containing: cytomegalovirus enhancer promoter (CMV, black), IgK signal peptide (IgK, pink) in frame with VP2 full ORF (VP2, blue) and gD93 (gD93, yellow). **b)** the sequence of the primers.

So derived construct was electroporated into HEK 293 T cells (as previously described)¹³⁰, cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and sovranatant of transiently transfected HEK 293T cells by western blotting.

Expression VP2 (marked with gD93)	
Cell fraction	Sovranatant
Undetectable	Undetectable

Figure 4.18. The VP2 expression (marked with gD93) in Mammalian cells and the present in cell extract and in sovranatant.

As demonstrated by Western (**Figure 4.8; 4.10; 4.12; 4.14; 4.16**) plgK-VP2-gD₃₆₁, plgK-VP2-gD₂₀₂, plgK-VP2-gD₁₈₁, plgK-VP2-gD₁₂₇, plgK-VP2-gD₁₀₆ expressed VP2 fused to the gD fragment (containing the anti-gD antibody epitope) into cell fraction but not into the medium. Although VP2 was provided of a functional signal peptide it was not enough to get VP2 sorted as a secreted form. plgKVP2 and plgK-VP2-gD₉₃ were not detected due to lack of gD epitope, thus showing that the minimal fragment containing gD epitope was gD₁₀₆. As a positive control for protein secretion and detection, Bovine Viral Diarrhea Virus (BVDV) glycoprotein E2 (gE2) fused to gD₁₀₆ epitope (plgK-E2- gD₁₀₆), was employed (figure 19), ensuring that gD₁₀₆ fragment did not have a detrimental effect for VP2 secretion.

plgK-E2-gD106

Strategy:

1. The fragment E2 (BVDV) of 1031bp was excised from plgKE2 by cutting with *HindIII* and *XhoI*.
2. This fragment was sub cloned into plgKVP2gD106 which was depleted of VP2 ORF by cutting with *HindIII* and *Sall* (*XhoI* and *Sall* generates compatible ends).

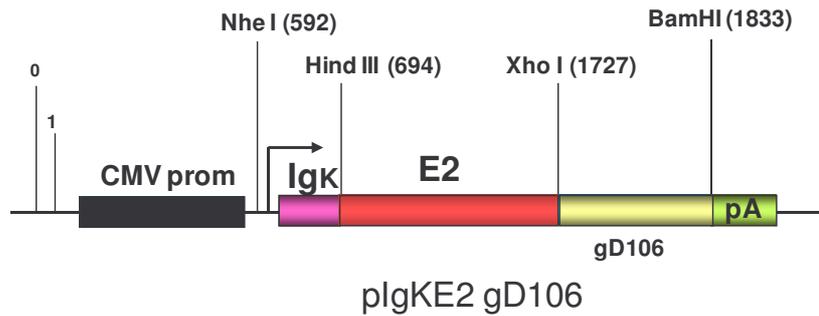


Figure 4.19. The pIgKVP2gD₁₀₆ construct containing: cytomegalovirus enhancer promoter (CMV, black), IgK signal peptide (IgK, pink) in frame with E2 ORF (E2, red), and gD₁₀₆ (gD₁₀₆, yellow).

So derived construct was electroporated into HEK 293 T cells, as previously described¹³⁰, and cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and sovranatant of transiently transfected HEK 293T cells by western blotting.

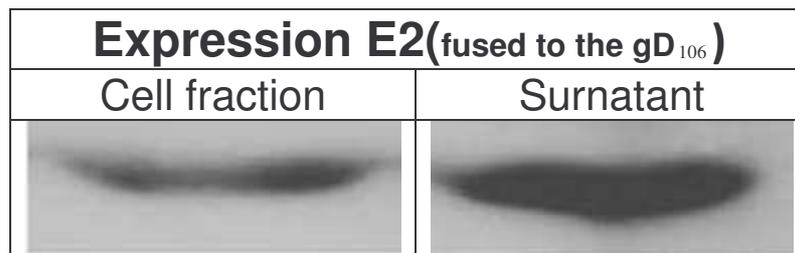


Figure 4.20. The E2, fused to the gD₁₀₆, epitope expression in Mammalian cells and the presence in cell extract and in sovranatant.

4.4 VP2 fragmentation

Basing on this information, gD₁₀₆ epitope was used as a general suitable VP2 tag for other constructs. In previous studies, it was demonstrated that BTV VP2 associates with protein cytoskeleton^{131,132} in both virus infected cells and in absence of other viral proteins. Further, the association of mature BTV particles with intermediate filaments was found to be driven by VP2 interaction with vimentin and, such interaction, contributed to virus egress. The determinant of vimentin localization was mapped to the N-terminus of the protein and deletions of amino acids between residues 65 and 114 was shown to disrupt VP2-vimentin association^{129,130,131}.

Because such VP2 association with intermediated filaments could be the cause of the missing VP2 secretion, several forms of truncated VP2 fused to IgK signal peptide were generated.

pIgK-VP2(4-1950)-gD106, containing the first 1946 bp of VP2 ORF and pIgK-VP2(975-2883)-gD106 containing the last 1907 bp were obtained using the primers Blue sense/VP2-tronc-anti and VP2-tronc-sense/Blue-anti respectively.

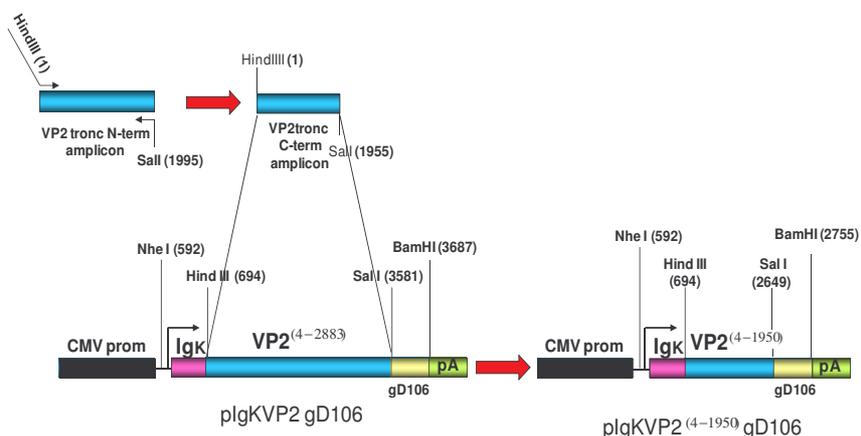
pIgK-VP2(4-1950)-gD106

Strategy:

VP2 fragment was generated by PCR with the primers Blu-sense and VP2tronc-anti. *HindIII* restriction site is contained in the 5' end of the amplicon just following sense primers, rather, *SalI* site is contained into the 3' end of the antisense primer.

So generated amplicon was cut with *HindIII* and *SalI* and the resulting 1946 pb fragment sub cloned into pIgKVP2gD106 which was depleted of the VP2 ORF cutting with *HindIII* and *SalI* (VP2 is in frame with gD fragment).

a)



b)

Primer name	Sequence
Blu-sense	5'-ccccga▼ agc ttg gag gag cta gcg att ccg att tat acg aat-3' HindIII
VP2-tronc-anti	3'-tgc tac ggg agc ctc cta aga ctt cat tgc cag ct▲g agcccc-5' Sall

Figure 4.21. a) The strategy to obtain plgKVP2(4-1950)gD106, the construct containing: cytomegalovirus enhancer promoter (CMV, black), IgK signal peptide (IgK, pink) in frame with VP2 full ORF (VP2, blue) and gD106 (gd106, yellow). **b)** the sequences of the primers

The construct was electroporated into HEK 293 T cells as previously described¹²⁹.

Cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and supernatant of transiently transfected HEK 293T cells by western blotting.

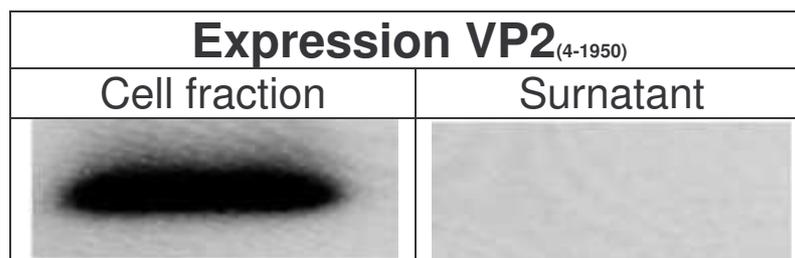


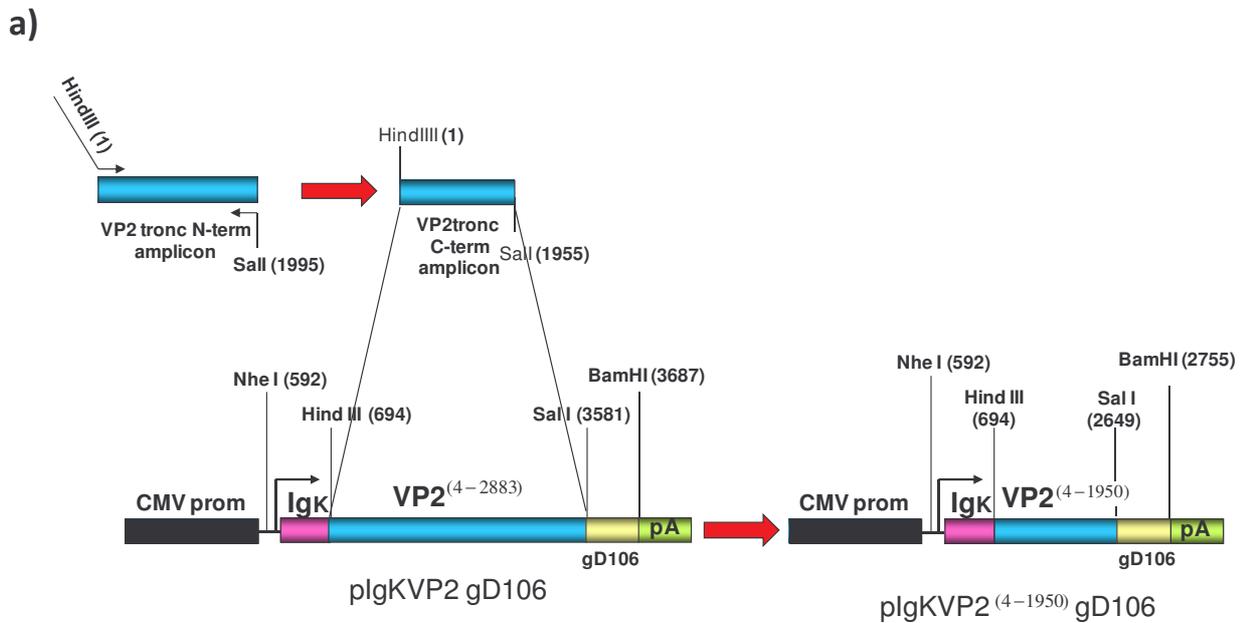
Figure 4.22. The VP2₍₄₋₁₉₅₀₎, fused to gD₁₀₆, epitope expression in Mammalian cells and the presence in cell extract and in supernatant.

plgK-VP2⁽⁹⁷⁵⁻²⁸⁸³⁾-gD106

Strategy:

A fragment of 1908 bp was generated by PCR with the primers VP2tronc-sense and Blu-anti. *HindIII* restriction site is contained in the 5' end of the amplicon just following the sense primers, rather, *Sall* site is contained into the 3' end of the antisense primer.

So generated amplicon was cut with *HindIII* and *Sall* and the resulting 1911 fragment sub cloned into plgKVP2gD₁₀₆ which was depleted of VP2 ORF cutting with *HindIII* and *Sall* (VP2 is in frame with gD fragment).



b)

Primer name	Sequence
VP2-tronc-sense	5'-ccccga ▼ agc ttg aac atc atg ggg ggc gga gta cat cgg aaa-3' HindIII
Blu-anti	3'-cta ctc tac aat tga ttc gac gag tta cat cag ct ▲ gagcccc-5' SalI

Figure 4.23 a) The strategy to obtain pIgKVP2⁽⁴⁻¹⁹⁵⁰⁾ gD₁₀₆, the construct containing: cytomegalovirus enhancer promoter (CMV, black), IgK signal peptide (IgK, pink) in frame with VP2 full ORF (VP2, blue) **b)** the sequences of the primers.

So derived construct was electroporated into HEK 293 T cells as previously described¹³⁰. and cells and culture medium were checked by western immuno-blotting using the anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and sovranatant of transiently transfected HEK 293T cells by western blotting.

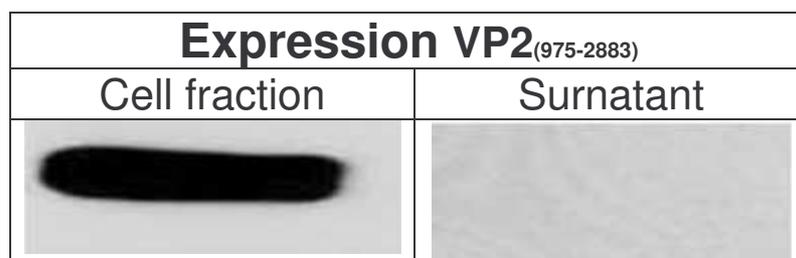


Figure 4.24. The VP2₍₉₇₅₋₂₈₈₃₎, fused to gD₁₀₆, epitope expression in Mammalian cells and the presence in cell extract and in sovranatant.

Although both constructs were well expressed into cell fraction, surprisingly none of them were able to bring production of secreted protein, even plgK-VP2₍₉₇₅₋₂₈₈₃₎-gD₁₀₆ lacking of the previously described vimentin binding residues.

Then, further truncations of VP2 ORF were made: **plgK-VP2₍₁₆₋₇₅₀₎-gD₁₀₆**, **plgK-VP2₍₇₂₀₋₁₄₂₅₎-gD₁₀₆**, **plgK-VP2₍₁₃₉₅₋₂₁₀₀₎-gD₁₀₆** and **plgK-VP2₍₂₀₇₀₋₂₈₈₃₎-gD₁₀₆**.

Strategy:

1. VP2 ORF was cut in 3 fragments by PCR amplification from pBRT7VP2 with four couples of primers containing an *HindIII* and a *Sall* restriction sites to the 5' and 3' end respectively.
2. VP2 fragments were cut with *HindIII/Sall* and in frame sub-cloned into *HindIII/Sall* plgKVP2gD₁₀₆.

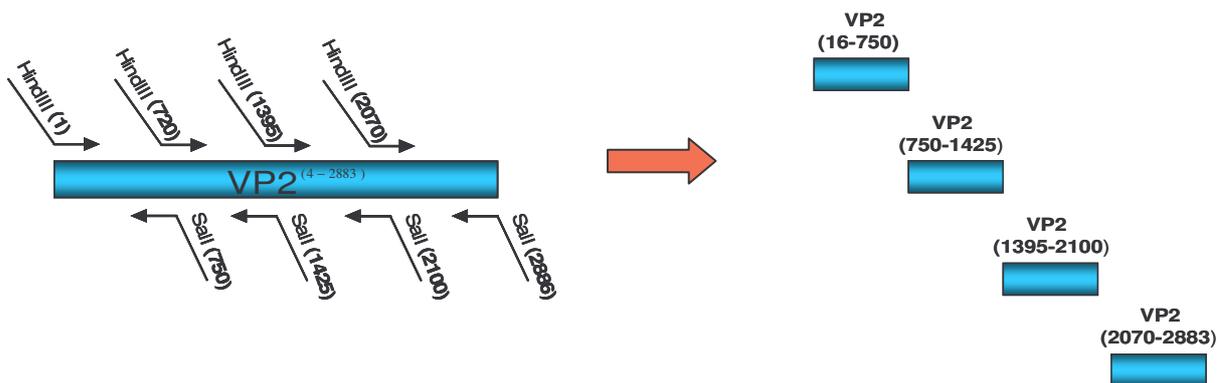
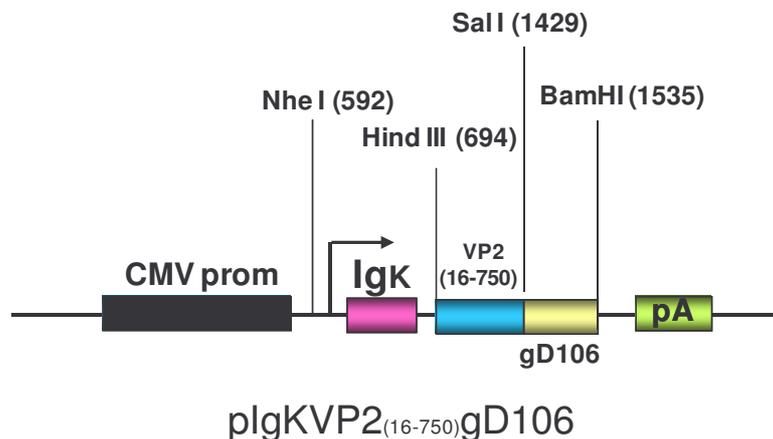


Figure 4.25 A full length of VP2 ORF and primer pairs to obtained the four fragment if VP2.

plgK-VP2₍₁₆₋₇₅₀₎-gD106

a)



b)

Primer name	Sequence
700 sense	5'-ccccga▼agc ttg att ccg att tat acg aat gta ttc cca gcg-3' HindIII
700 anti	3'-gtt caa gct taa ctt ccc ttc tgc gga aac cag ct▲gagcccc-5' Sall

Figure 4.26 Diagram (not to scale) showing the construct, pIgK-VP2₍₁₆₋₇₅₀₎-gD₁₀₆; containing: cytomegalovirus enhancer promoter (CMV, black), IgK signal peptide (IgK, pink) in frame with VP2 fragments (VP2, blue) and gD₁₀₆ ectodomain (gD, yellow). **b)** The sequences of the primers

So derived construct was electroporated into HEK 293 T cells as previously described¹³⁰ and cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and sovranatant of transiently transfected HEK 293T cells by western blotting.

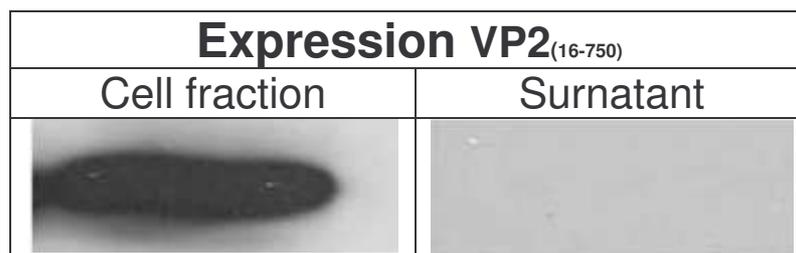
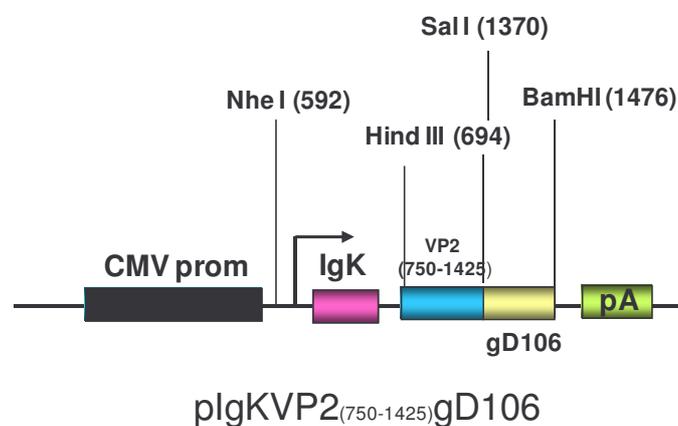


Figure 4.27 The VP2₍₁₆₋₇₅₀₎, fused to gD₁₀₆, epitope expression in Mammalian cells and the present in cell extract and in sovranatant.

pIgK-VP2₍₇₅₀₋₁₄₂₅₎-gD106

a)



b)	Primer name	Sequence
	1400 sense	5'-ccccga▼ agc ttg caa gtt cga att gaa ggg aag acg cct ttg-3' HindIII
	1400 anti	3'-tgc ctc tac ttc ttg gtt taa taa gtt tcg cag ct▲gagcccc -5' Sall

Figure 4.28 Diagram (not to scale) showing the construct, pIgK-VP2₍₇₅₀₋₁₄₂₅₎-gD₁₀₆; containing: cytomegalovirus enhancer promoter (CMV, black), IgK signal peptide (IgK, pink) in frame with VP2 fragments (VP2, blue) and gD₁₀₆ ectodomain (gD, yellow). **b)** The sequences of the primers

The obtained construct was electroporated into HEK 293 T cells as previously described¹³⁰.

Cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and surnatant of transiently transfected HEK 293T cells by western blotting.

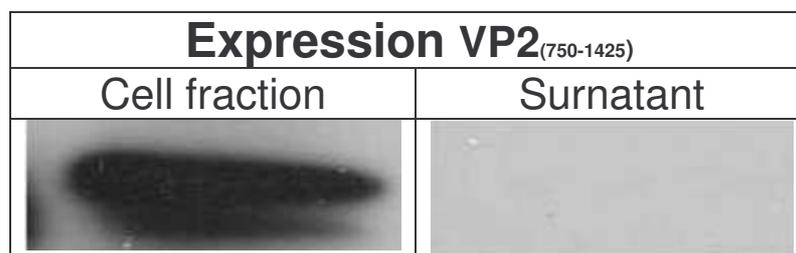
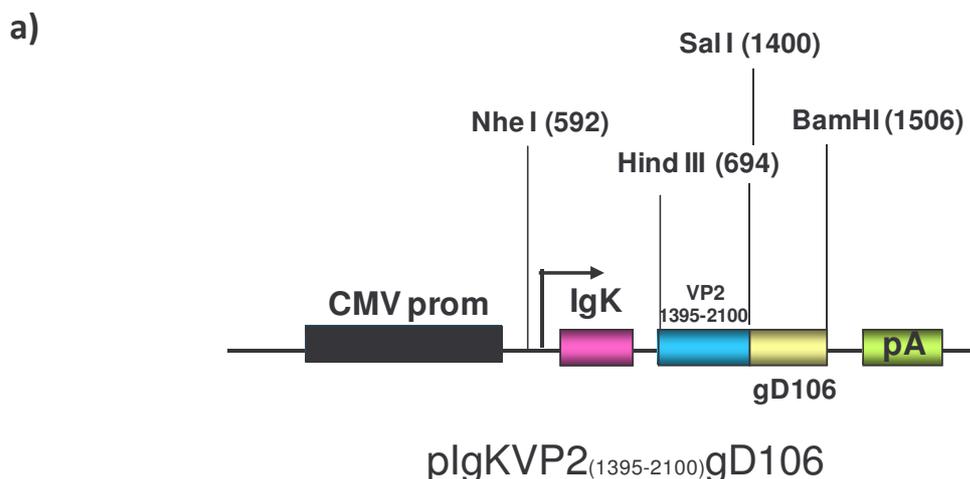


Figure 4.29 The VP2₍₇₅₀₋₁₄₂₅₎, fused to gD₁₀₆, epitope expression in Mammalian cells and the presence in cell extract and in sovranatant.

pIgK-VP2₍₁₃₉₅₋₂₁₀₀₎-gD106



Primer name	Sequence
2100 sense	5'-ccccga▼ agc ttg acg gag atg aag aac caa att att caa agc-3' HindIII
2100 anti	3'-tga tgg ctc cgt tcc acc tgc gaa aag ttt cag ct▲ gagcccc-5' Sall

Figure 4.30. Diagram (not to scale) showing the construct, pIgK-VP2(1395-2100)-gD106; containing: cytomegalovirus enhancer promoter (CMV, black), IgK signal peptide (IgK, pink) in frame with VP2 fragments (VP2, blue) and gD106 ectodomain (gD, yellow). **b)** The sequences of the primers.

So derived construct was electroporated into HEK 293 T cells as previously described¹²⁹ cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into the cell fraction and sovranatant of transiently transfected HEK 293T cells by western blotting.

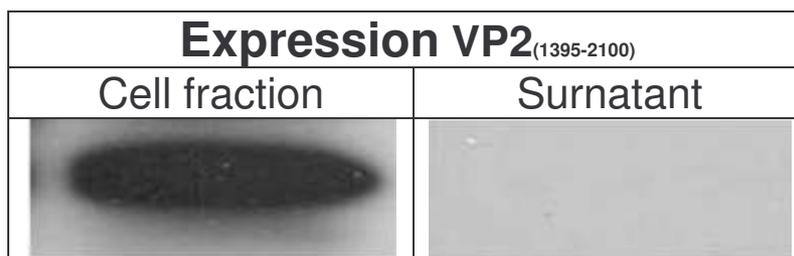
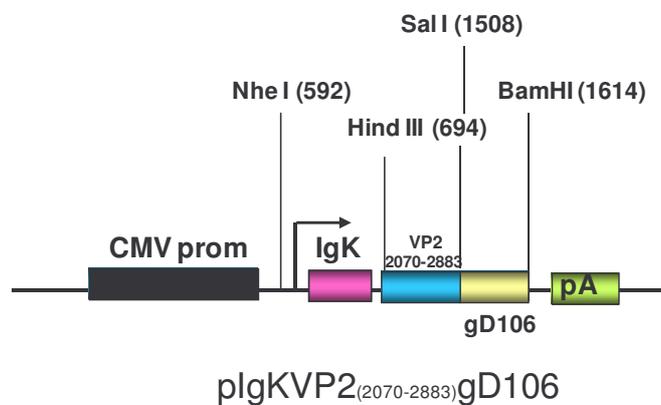


Figure 4.31 The VP2₍₁₃₉₅₋₂₁₀₀₎, fused to gD₁₀₆, epitope expression in Mammalina cells and the presence in cell extract and in sovranatant.

pIgK-VP2₍₂₀₇₀₋₂₈₈₃₎-gD106

a)



b)	Primer name	Sequence
	2800 sense	5'-ccccga ▼agc ttg act acc gag gca agg tgg acg ctt ttc aaa-3' HindIII
	Blu-anti	3'-cta ctc tac aat tga ttc gac gag tta cat cag ct▲gagcccc-5' Sall

Figure 4.32 Diagram (not to scale) showing the construct, plgK-VP2₍₂₀₇₀₋₂₈₈₃₎-gD₁₀₆; containing: cytomegalovirus enhancer promoter (CMV, black), IgK signal peptide (IgK, pink) in frame with VP2 fragments (VP2, blue) and gD₁₀₆ ectodomain (gD, yellow). **b)** The sequences of the primers.

So derived construct was electroporated into HEK 293 T cells as previously described¹²⁹ and cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and sovranatant of transiently transfected HEK 293T cells by western blotting

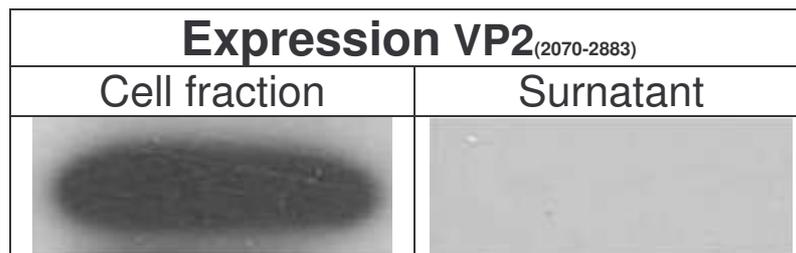


Figure 4.33 The VP2₍₂₀₇₀₋₂₈₈₃₎, fused to gD₁₀₆, epitope expression in Mammalian cells and the presence in cell extract and in sovranatant.

4.5 VP2chimeric peptide generation

Reasoning on the fact that BVDV gE2 ectodomain was very well expressed and secreted by plgK-E2-gD₁₀₆¹³³, IgK-gE2 peptide was exploited as a leader peptide to get VP2, derived fragments secreted into the medium of transfected cells.

Strategy:

E2 ORF fragment was obtained by plgKE2gd106 cut with *HindIII* and *XhoI*. *HindIII* site is contained in the 5' end, rather, site *XhoI* is contained into the 3' end.

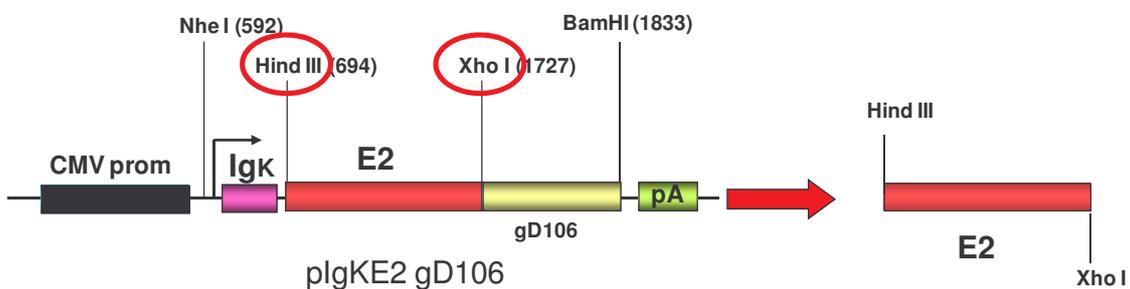


Figure 4.34 The strategy to obtain HindIII/E2/XhoI from PlgKE2gD106 construct.

The resulting 1033 bp fragment was sub cloned into plgKVP2gD₁₀₆, plgKVP2₇₀₀gD₁₀₆, plgKVP2₁₄₀₀gD₁₀₆, plgKVP2₂₁₀₀gD₁₀₆ and plgKVP2₂₈₀₀gD₁₀₆ also cut with *HindIII*.

It is obtained: plgKE2-VP2-gD₁₀₆, plgKE2-VP2₇₀₀gD₁₀₆, plgKE2-VP2₁₄₀₀gD₁₀₆, plgKE2-P2₂₁₀₀gD₁₀₆ and plgKE2-VP2₂₈₀₀gD₁₀₆.

plgK-E2(4-2883)-gD106

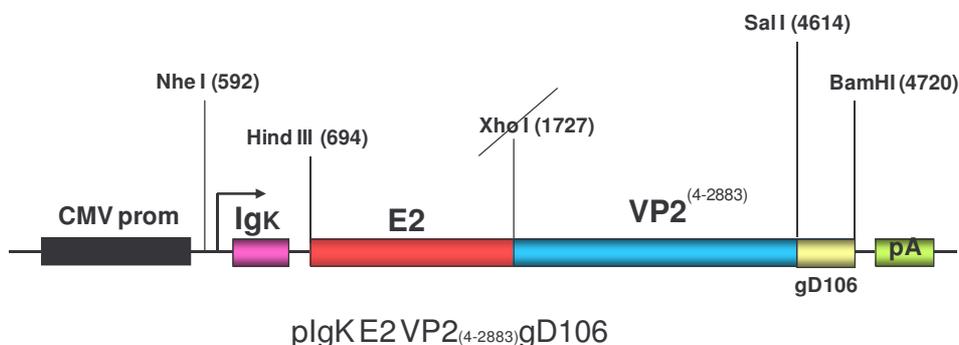


Figure 4.35 Diagram (not to scale) of the constructs, plgK-E2-VP2₍₄₋₂₈₈₃₎-gD₁₀₆; containing: cytomegalovirus enhancer promoter (CMV, black), IgK signal peptide (IgK, pink) in frame with E2 fragments (E2, red), VP2 fragments (VP2, blue) and gD₁₀₆ ectodomain (gD, yellow).

This construct was electroporated into HEK 293 T cells as previously described¹²⁹ and cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and sovranatant of transiently transfected HEK 293T cells by western blotting.

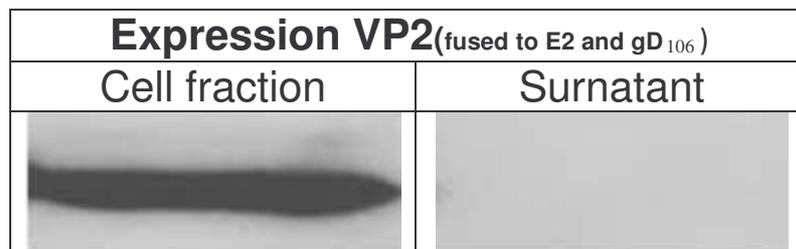


Figure 4.36 The VP2, fused to E2 and gD₁₀₆, epitope expression in Mammalian cells and the presence in cell extract and in sovranatant.

plgK-E2⁽¹⁶⁻⁷⁵⁰⁾-gD106

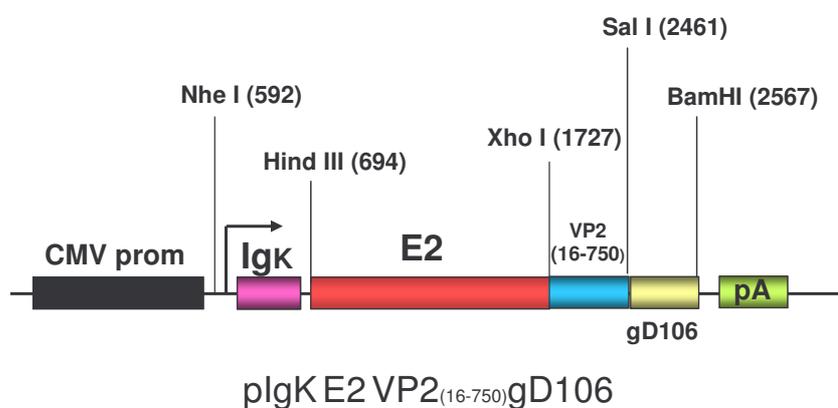


Figure 4.37 Diagram (not to scale) of the constructs, plgK-E2-VP2₍₁₆₋₇₅₀₎-gD106; containing: cytomegalovirus enhancer promoter (CMV, black), IgK signal peptide (IgK, pink) in frame with E2 fragments (E2, red), VP2 fragments (VP2, blue) and gD106 ectodomain (gD, yellow).

This construct was electroporated into HEK 293 T cells as previously described¹³⁰ and cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and sovranatant of transiently transfected HEK 293T cells by western blotting.

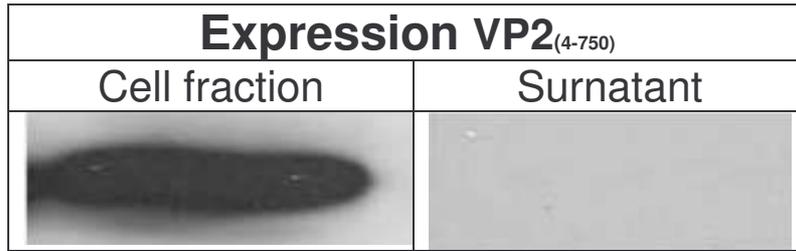


Figure 4.38 The VP2, fused to E2 and gD₁₀₆, epitope expression in Mammalian cells and the presence in cell extract and in sovranatant

pIgK-E2⁽⁷⁵⁰⁻¹⁴²⁵⁾-gD106

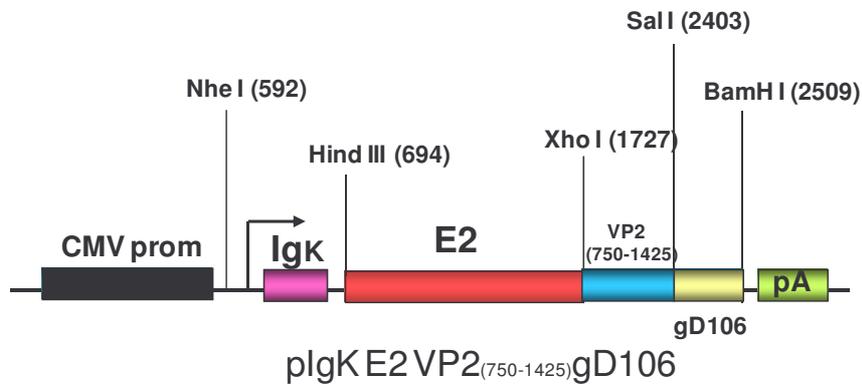


Figure 4.39 Diagram (not to scale) of the constructs, pIgK-E2-VP2₍₇₅₀₋₁₄₂₅₎-gD₁₀₆; containing: cytomegalovirus enhancer promoter (CMV, black), IgK signal peptide (IgK, pink) in frame with E2 fragments (E2, red), VP2 fragments (VP2, blue) and gD₁₀₆ ectodomain (gD, yellow).

So derived construct was electroporated into HEK 293 T cells as previously described¹³⁰ and cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and sovranatant of transiently transfected HEK 293T cells by western blotting.

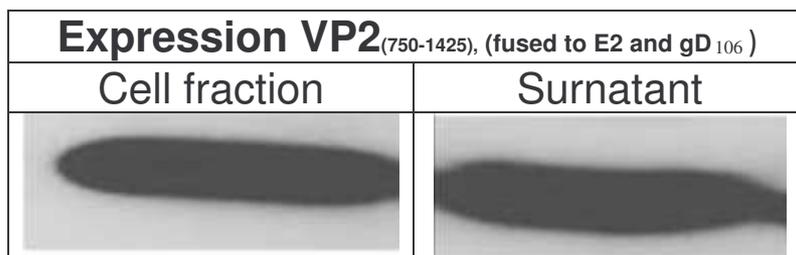


Figure 4.40 The VP2₍₇₅₀₋₁₄₂₅₎, fused to E2 and gD₁₀₆, epitope expression in Mammalian cells and the presence in cell extract and in sovranatant.

pIgK-E2(1395-2100)-gD106

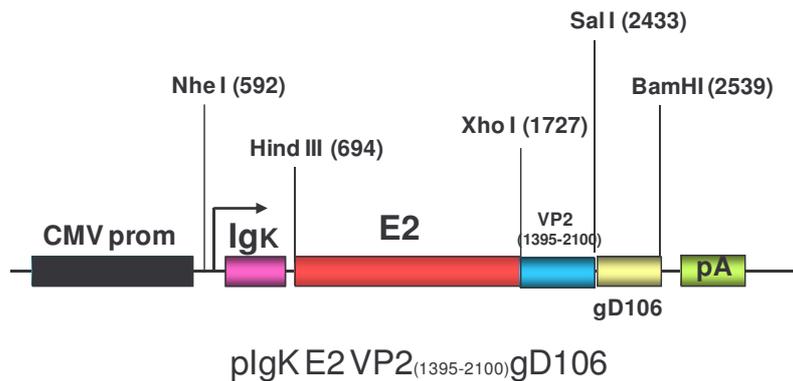


Figure 4.41 Diagram (not to scale) of the constructs, pIgK-E2-VP2₍₁₃₉₅₋₂₁₀₀₎-gD₁₀₆; containing: cytomegalovirus enhancer promoter (CMV, black), IgK signal peptide (IgK, pink) in frame with E2 fragments (E2, red), VP2 fragments (VP2, blue) and gD₁₀₆ ectodomain (gD, yellow).

So derived construct was electroporated into HEK 293 T cells as previously described¹³⁰ and cells and culture medium were checked by western immuno-blotting using anti-gD antibody for analysis of VP2 expression.

Expression was tested into cell fraction and sovranatant of transiently transfected HEK 293T cells by western blotting.

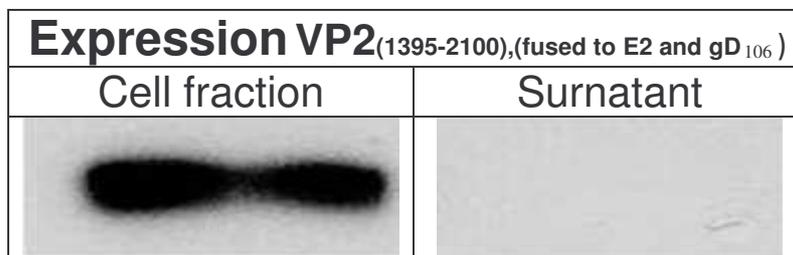


Figure 4.42 The VP2₍₁₃₉₅₋₂₁₀₀₎, fused to E2 and gD₁₀₆, epitope expression in Mammalian cells and the presence in cell extract and in sovranatant.

pIgK-E2(2070-2883)-gD106

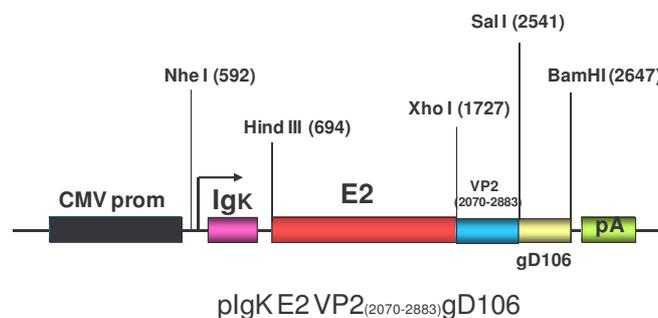


Figure 4.43 Shows (not to scale) the constructs, pIgK-E2-VP2₍₂₀₇₀₋₂₈₈₃₎-gD₁₀₆; containing: The cytomegalovirus enhancer promoter (CMV, black), the IgK signal peptide (IgK, pink) in frame with E2 fragments (E2, red), VP2 fragments (VP2, blue) and the gD₁₀₆ ectodomain (gD, yellow).

pIgKE2VP2₍₂₀₇₀₋₂₈₈₃₎gD₁₀₆ was electroporated into HEK 293 T cells as previously described¹³⁰ and cells and culture medium were checked by western immuno-blotting using the anti-gD antibody for analysis of VP2 expression.

Expression was tested into the cell fraction and sovranatant of transiently transfected HEK 293T cells by western blotting.



Figure 4.44 Shows VP2₍₂₀₇₀₋₂₈₈₃₎, fused to the E2 and gD₁₀₆, epitope espression in Mammalina cells and the present in extract or in sovranatant.

pIgK-E2-VP2₍₇₂₀₋₁₄₂₅₎-gD₁₀₆ and pIgK-E2-VP2₍₂₀₇₀₋₂₈₈₃₎-gD₁₀₆ successfully expressed and secreted the VP2 fragments, whereas for the other construct, although expression was achieved, no protein secretion was detected.

4.6 BoHV-4 U Δ ORF50 IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶

In order to generate a recombinant virus expressing IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ chimeric peptide, we have chosen a mutant BoHV-4 U Δ ORF50kanaGalk strain¹³⁴ in which IE2 locus is duplicated and one of them is inactivated by the insertion of 2004 bp KanaGalk DNA sequence stuffer double selecting cassette¹³⁵ in ORF50 Exon 2.

A re-targeting construct, pLEFT/IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶/RIGHT, in which IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ expression cassette is flanked by two BoHV-4 IE2 gene homologous region sequences, was generated.

Strategy:

The AseI/MluI fragment from plgKE₂VP₂²⁸⁰⁰gD¹⁰⁶, which includes the complete sequence of IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ expression cassette with his CMV promoter and polyadenylation site, was sub-cloned in pORF50KanaGalk¹³⁴ after a KpnI/PstI double digestion end by a blunt end reaction.

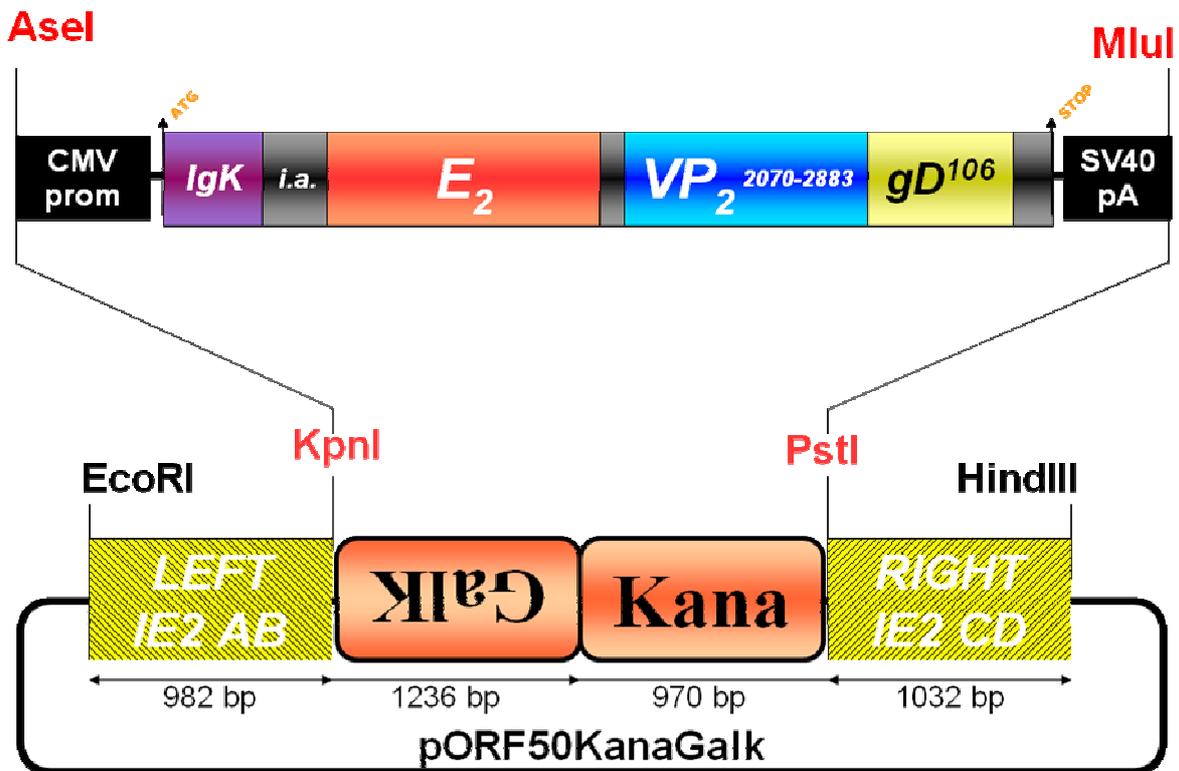


Figure 4.45 Shows IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ expression cassette, after AseI/MluI double digestion, sub-cloned in pORF50KanaGalk, after KpnI/PstI double digestion, by Blunt end reaction.

So has been obtain pORF50/IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ (pLEFT/ IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶/RIGHT):

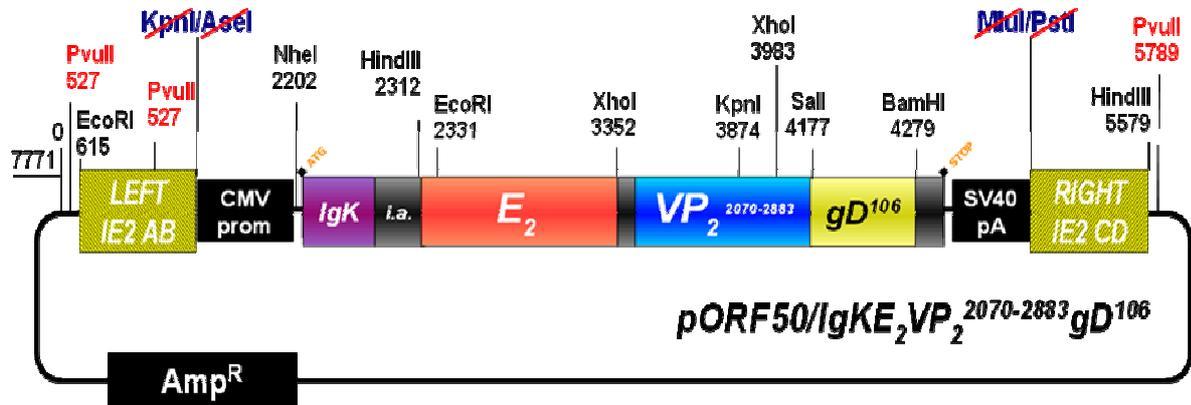


Figure 4.46 Shows the new plasmid construct pORF50/IgKE₂VP₂⁰⁷⁰⁻²⁸⁸³⁰gD¹⁰⁶ in which IgKE₂VP₂²⁰⁷⁰⁻²⁸³gD¹⁰⁶ expression cassette is flanked by two BoHV-4 IE2 gene homologous region sequences Left (IE2 AB) and Right (IE2CD).

Before using the pORF50/IgKE₂VP₂²⁸⁰⁰gD¹⁰⁶ plasmid for recombination, we made sure that the introduction of homologous sequence did not alter the frame of coding sequence or the expression cassette efficiency. So pORF50/IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ plasmid was transiently transfected by electroporation into HEK 293 T cells and, 24h p.t., expression was tested into cell fraction and sovranatant of transiently transfected HEK 293 T cells by western blotting, using anti-gD antibody.

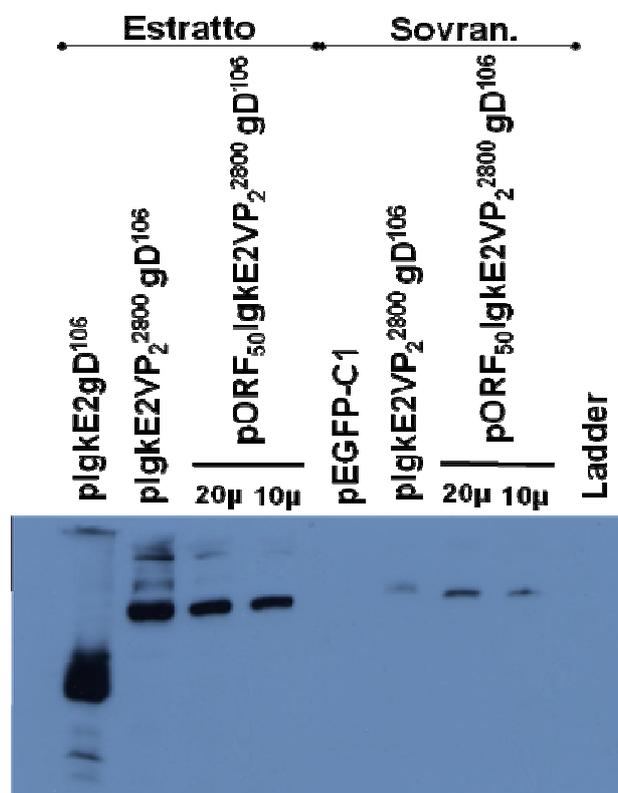


Figure 4.47 Western immunoblotting of pORF50/IgKE₂VP₂⁰⁷⁰⁻²⁸⁸³⁰gD¹⁰⁶-transfected cell extracts and supernatants. pIgKE₂gD¹⁰⁶ and pIgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶-transfected cell extracts and supernatants were used as a positive controls; pEGFP-C1-transfected cell supernatant was used as a negative control.

The generated re-targeting vector LEFT/ IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶/RIGHT was excised from the plasmid backbone through PvuII restriction enzyme digestion and the fragment of ≈4428bp was used for heat inducible homologous recombination in SW102 E. coli containing pBAC BoHV-4 U ΔORF50KanaGalk genome targeted into the ORF50 locus with KanaGalk sequence stuffer double selecting cassette.

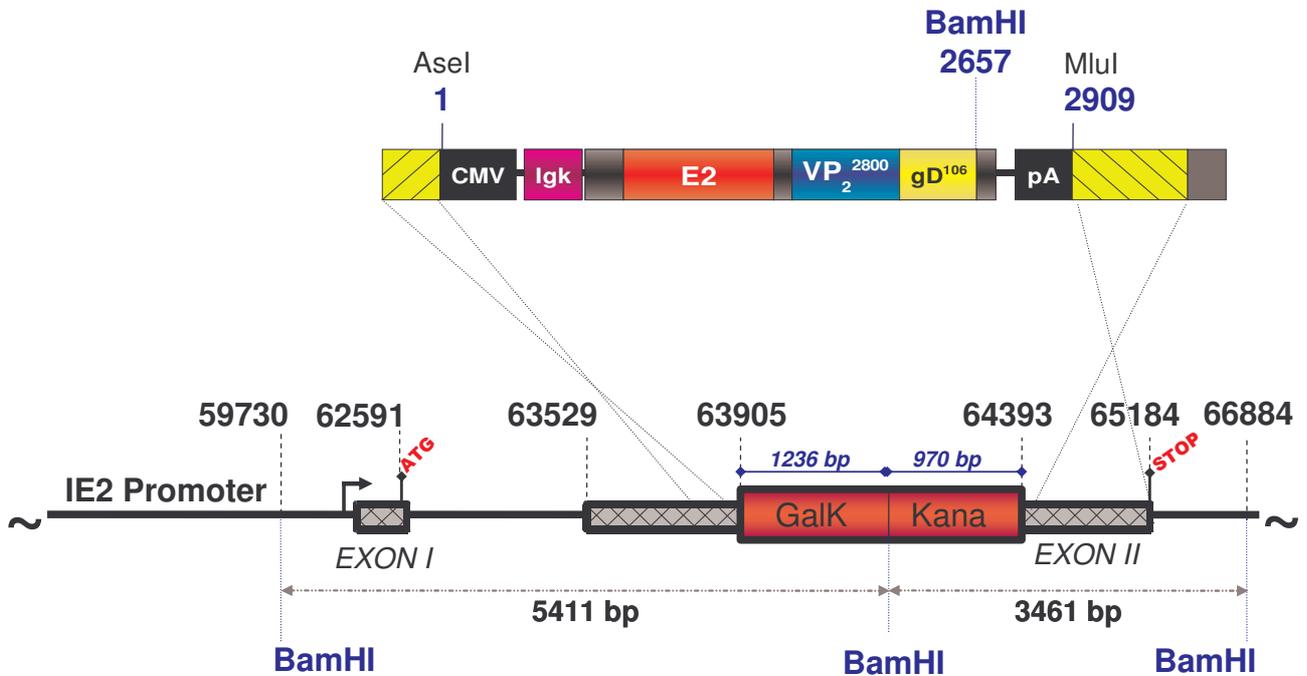


Figure 4.48 Diagram not to scale shows the re-targeting by heat-inducible homologous recombination in SW102 containing pBAC-BoHV-4 U ΔORF50 KanaGalk, where Kana/Galk cassette was removed and replaced with IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ expression cassette .

So pBAC BoHV-4 U ΔORF50-IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ it's obtained:

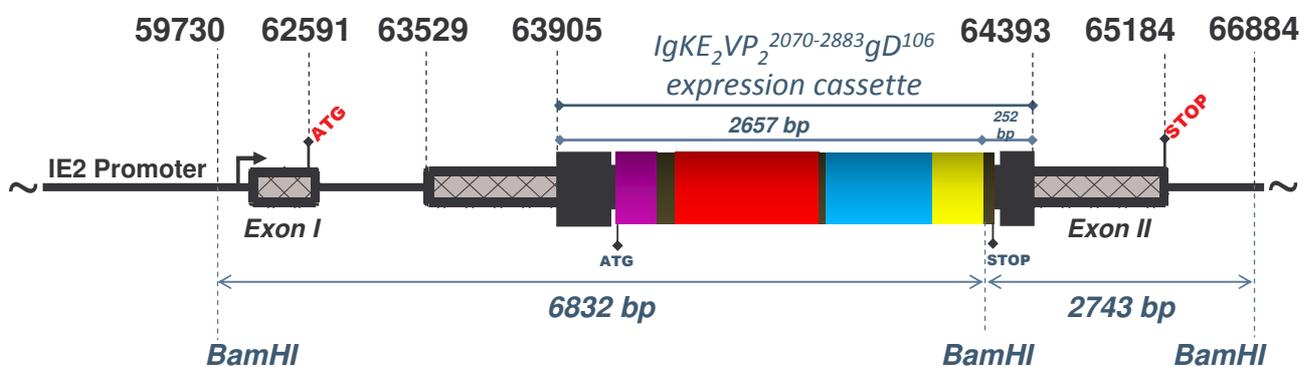


Figure 4.49 Diagram shows not to scale the final recombinant BAC BoHV-4 U ΔORF50-IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶

Re-targeting was performed to the same site to replace the KanaGalk cassette with the IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ cassette, through a negative selection on deoxygalactose (DOG) minimal plates and a negative selection with medium containing kanamycin. Only clones surviving in medium containing chloramphenicol but dying in medium containing kanamycin were analyzed to verify the integration of IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ cassette. This procedure allowed the isolation of positive clones for the right targeting, BoHV-4 U ΔORF50-IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶.

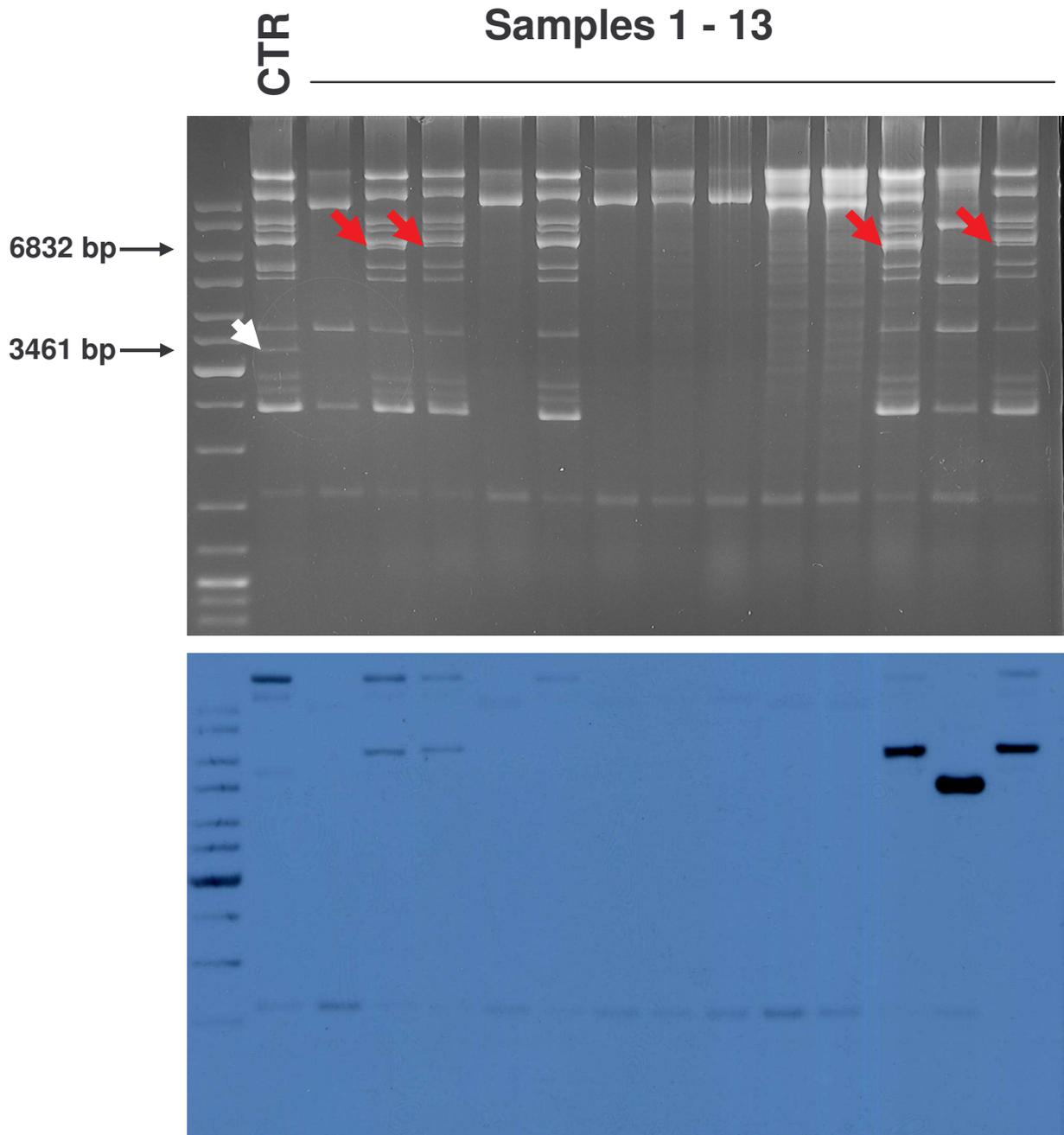


Figure 4.50 Colonies tested by BamHI restriction enzyme analysis: agar gel electrophoresis and Southern blotting showing the right re-targeting, where the 3490 bp band (targeting (CTR), indicated by white arrow) disappeared and a 7018 bp band (re-targeting, indicated by red arrows), detected by Southern blotting with a gE2 probe, appeared.

To reconstitute the infectious virus, pBAC BoHV-4 U Δ ORF50-IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ was electroporated into BEK or BEKcre cells to excise the BAC cassette. Viable virus was obtained in both case and the depletion of the floxed BAC cassette in the progey virus was assessed by loss of the green fluorescence in the viral plaques in consequence of the excision of the CMV-GFP expression cassette conained in te BAC plasmid backbone. When HEK 293T cells were infected with recombinant BoHV-4 U Δ ORF50-IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶, they robustly expressed and secreted into the medium the chimeric peptide IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶.

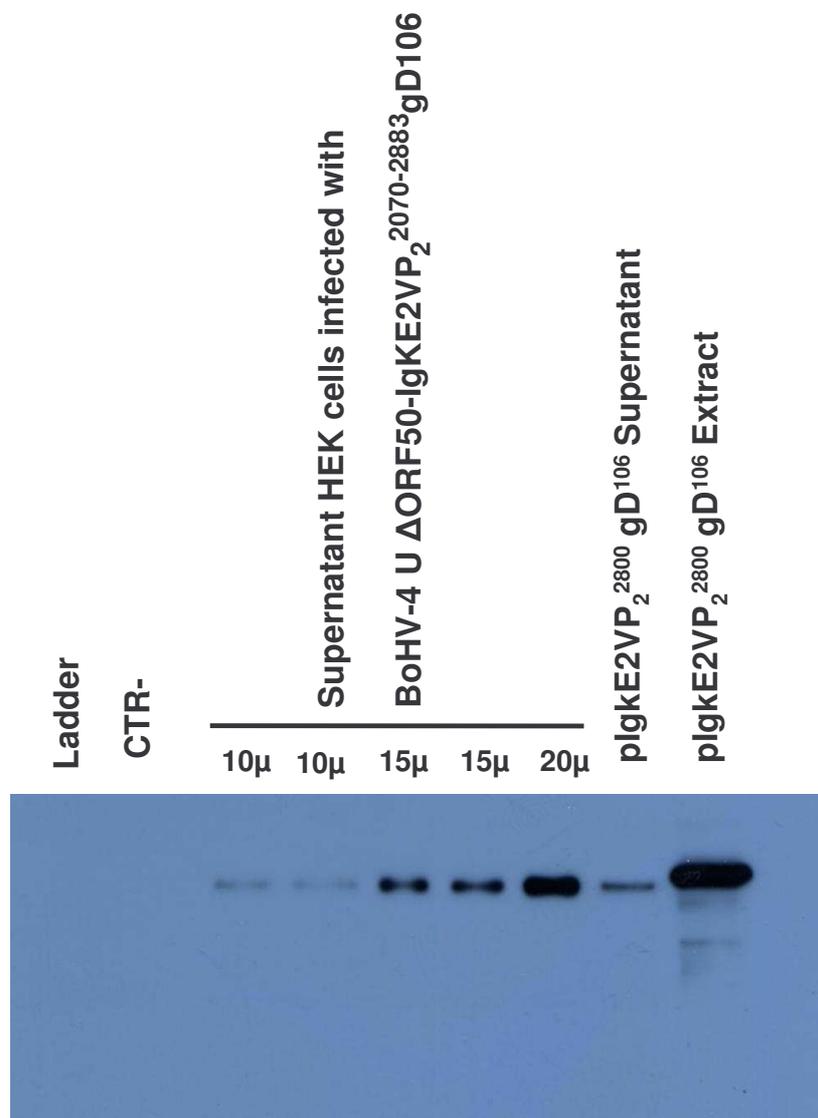


Figure 4.51 Western immunoblotting of BoHV-4 U Δ ORF50-IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶-infected HEK cells supernatant. Supernatant of not-infected HEK cells was used as negative control. Supernatant and extract of plgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶-transfected HEK cells were used as a positive controls.

5 CONCLUSIONS

VP2, the outermost viral protein, shows an architecturally complex structure, which corresponds to a multiplicity of functions.

VP2 is responsible for host cell receptor binding, but, probably, also plays a role in virus egress from infected cells as it binds the NS3, a viral non structural protein that acts as a molecular bridge between the virus particle and cellular export and release factors, and the vimentin intermediate filaments. Thus, VP2 is important to both the infectivity and egress of BTV particles⁸⁴.

The great interest in this viral protein is also due to its ability to elicit virus-neutralizing antibody and serotype specificity.

Due to its biochemical characteristics, VP2 is proven to be difficult to express as a soluble form in mammalian cells and, currently, the insect cell-baculovirus expression system is a powerful platform to rapidly produce high levels of recombinant VP2 protein.

Now we proposed a mammalian cell-BoHV₄ viral vector based expression system to obtain high levels of recombinant BTV-8 VP2 protein in a soluble form.

First, VP2 ORF was re-designed to allow protein secretion into the medium of transfected cells:

1. The full length of VP2 was sub-cloned in frame with the immunoglobuline kappa chain (I_gk) signal peptide to obtain plgkVP2, the first VP2 expression plasmid vector.
2. In order to monitor expression/secretion of VP2 by Western blotting, due to the lack of a suitable monoclonal antibody against BTV-8 VP2, a tag soluble epitope of Bovine herpesvirus 1(BoHV-1) glycoprotein D (gD) was mapped and we found that the smallest gD fragment that still retains the epitope of interest is gD¹⁰⁶. Based on this information, gD¹⁰⁶ peptide was used as a VP2 tag and we obtain plgkVP2gD¹⁰⁶.
3. plgkVP2gD¹⁰⁶ was transfected in HEK cells and, successively, cell extract and supernatant was analysed by Western blotting; we found that plgkVP2gD¹⁰⁶ was very well expressed but not secreted, showing that the introduction of a heterologous signal peptide in the N-terminus of VP2 is not sufficient to induce its secretion. Since plgkVP2gD¹⁰⁶ is expressed but not secreted, positive control plgKE2gD¹⁰⁶, well expressed and secreted, ensures that the gD¹⁰⁶ fragment did not have a detrimental effect for VP2 secretion.
4. Since association of VP2 with vimentin intermediate filaments could be the cause of the lack of VP2 secretion, several forms of truncated VP2 in frame upstream with

Igk signal peptide and downstream with gD¹⁰⁶ sequence tag were generated. Although all constructs were well expressed in the cell fraction, surprisingly none of them were able to make secreted protein, including plgkVP2⁹⁷⁵⁻²⁸⁸³gD¹⁰⁶ lacking vimentin binding sequence. Thus, we deduced that VP2 interaction with intracellular proteins or other cellular elements must be much more complex than the simple interaction with vimentin. In fact, deletion of the putative VP2 vimentin interacting portion in the VP2 amino terminal was not enough to ensure the secretion of the rest of the protein.

5. Based on the fact that the BVDV gB ectodomain was well expressed and secreted by plgkE2gD¹⁰⁶, the IgkE2 was exploited as a leader sequence to get VP2 and derived fragments secreted into the transfected cell medium. We found that plgkE2VP2⁷²⁰⁻¹⁴²⁵gD¹⁰⁶ and plgkE2VP2²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ successfully expressed and secreted the VP2 fragments, so we deduced that the strategy to sandwich an un-secreted peptide between two secreted peptides, represent a useful approach to get secreted the un-secreted peptide.

Finally, in order to generate a powerful vector system to rapidly produce high levels of VP2 fragment, we inserted IgkE2VP2²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ expression cassette into the exon 2 of IE2 gene of BoHV-4 U ΔORF50KanaGalK strain, in which IE2 gene is duplicated and one of them is inactivated by the insertion of 2004 bp KanaGalK DNA sequence stuffer double selecting cassette¹³⁴, cloned as a bacterial artificial chromosome. Thus, we generated BoHV-4 U ΔORF50-IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ recombinant virus. HEK cells infected with BoHV-4 U ΔORF50-IgKE₂VP₂²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶ secret into the medium high levels of IgkE2VP2²⁰⁷⁰⁻²⁸⁸³gD¹⁰⁶.

Therefore, we now provide a new powerful tool to generate suitable amounts of VP₂²⁰⁷⁰⁻²⁸⁸³ fragment to the development of new serodiagnostic assays and successful vaccines.

BIBLIOGRAPHY

1. **Cox H. R.**, (1954) Bluetongue. *Bacteriol Rev.* **18**,239-253.
2. **Erasmus, B.J.**, (1975). Bluetongue in sheep and goats. *Aust. Vet. J.*, **51**, 165-70.
3. **Mellor P., Baylis M. & Mertens P.** (2009). Bluetongue. *First edition, London, Academic Press.*, chapter 2.
4. **Maclachlan N. J., Drew C. P., Darpel K. E and Worwa G.**. 2009. The Pathology and Pathogenesis of Bluetongue. *J. of Comparative Pathology.* **141**, 1-16.
5. **Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Bal L.A.**, (2005) Virus Taxonomi, Classification and nomenclature. *London, Elsevier Accademic Press, eight edition.*
6. **Chaignat V, Worwa G, Scherrer N, Hilbe M, Ehrensperger F, Batten C, Cortyen M, Hofmann M, Thuer B.**, (2009) Toggenburg Orbivirus, a new bluetongue virus: initial detection, first observations in field and experimental infection of goats and sheep. *Vet Microbiol.* **138**:11-9.
7. **Hofmann M. A., Renzullo S., Planzer J., Mader M.**, (2010) Detection of Toggenburg Orbivirus by a segment 2-specific quantitative RT-PCR. *J. Virol. Methods.* **165**:325-9.
8. **Tabachnick W.J.**, (2004). Culicoides and the global epidemiology of bluetongue virus infection. *Vet Ital.* **40**:144-150.
9. **Wilson A. & Mellor P.** (2009) Bluetongue in Europe: past, present and future. *Phil. Trans. R. Soc. B*, **364**, 2669-2681.
10. **Wilson A. & Mellor P.** (2008) Bluetongue in Europe: vectors, epidemiology and climate change. *Parasitology Research*, **103**, S69-S77.
11. <http://www.salute.gov.it/alimenti/sanita/sanApprofondimento.jsp?lang=italiano&label=mal&id=224&dad=s>
12. **N.J. MacLachla.** (2004) Bluetongue: pathogenesis and duration of viraemia. *Vet. Ital.*, **40**, 462-467.

13. **Mellor P., Baylis M. & Mertens P.** (2009). Bluetongue. *First edition, London, Academic Press.*, chapter 1.
14. Report of Cattle and Sheep Disease Commission, 1876-1877, p.xvi and 189.
15. **Henning, M. W.** (1949) Blue-Tongue, Bloutong. Animal diseases in South Africa. *2nd ed. Central News Agency, Ltd., South Africa.*
16. **Hutcheon D.** (1881) Fever or epizootic catarrh. *Rept. Coll. Vet. Surg. for 1880, 12.*
17. **Hutcheon D.** (1902) Malarial Catarrhal fever of sheep. (1902) *Vet. Rec. 14*, 629-633.
18. **Spreull, J.** (1905) Malarial catarrhal fever (bluetongue) of sheep in South Africa. *J. Comp. Pathol. Therap., 18*, 321-337.
19. **Theiler, A.** (1906) Blue-tongue in sheep. *Ann. Rept. Director Agric., Transvaal, for 1904-1905*, 110-121.
20. **Mellor P. S., Carpenter S., Harrup L., Baylis M., Mertens P. P. C.,** (2008) Bluetongue in Europe and the Mediterranean Basin: History of occurrence prior to 2006. *Preventive Veterinary Medicine 87*,4-20.
21. **Gambles R.,** (1949) Bluetongue of sheep in Cyprus. *J C omp Pathol, 59*, 176-190.
22. **Manso-Ribeiro J., Rosa-Azevedo J., Noronha F., Braco-Forte-Junior M., Grave-Preiera C., Vasco-Fernandes M.** (1957) Fievre Catarrhale du mouon (Bluetongue). *Bulletin de l'Office International dses Epzooties, 48*, 350-367.
23. **Hardy W.T., Price D.A.,** (1952) Soremuzzle of sheep. *J Am Vet Med Assoc., 120*, 23-25.
24. **Sapre S. N.,** (1964) An outbreak of Bluetongue in goat and sheep. *Vet. Rev., 15*, 75-80.
25. **St George T. D., Standfast H. A., Cybinski D. H., Dyce A. L., Muller M. D., Doherty R. L., Carley J. D., Filippic C., Frazier C. L.,** (1978) The isolation of Bluetongue virus from Culicoides collected in Northern Territory of Australia. *Aust. Vet. 43*, 153-154.
26. **Vasalos M.,** (1980) Cas de fievre Catarrhal du mouton dan l'ile Lesbos (Greek). *Bulletin de l' Office International des Epzooties, 92*, 547.

27. **Anon**, (1998) Bluetongue in Greece: confirmation of the diagnosis. *Office International des Epizooties Disease information*, **11**, 166-1667.
28. **Osmani A, Murati B, Kabashi Q, Goga I, Berisha B, Wilsmore AJ, Hamblin C.**, (2004) Evidence for the presence of bluetongue virus in Kosovo between 2001 and 2004. *Vet Rec.*, **158**, 393-396.
29. **Breard E., Sailleau C., Nomikou K., Hamblin C., Mertens P. P., Mellor P. S., El Harrak M., Zientara S.**, (2007) Molecular epidemiology of bluetongue virus serotype 4 isolated in the Mediterranean Basin between 1979 and 2004. *Virus Res.*, **125**, 191-197.
30. **Mertens P. P., Maan N. S., Prasad G., Samuel A. R., Shaw A. E., Potgieter A. C., Anthony S. J., Maan S.**, (2007) Design of primers and use of RT-PCR assays for typing European bluetongue virus isolates: differentiation of field and vaccine strains. *J. Gen. Virol.*, **88**, 811-2823.
31. **Anon**. (2006) Bluetongue virus in the Netherlands identified as serotype 8 by Institute for Animal Health. *IAH press release, 2006-08-29. Institute for Animal Health*.
32. EFSA 2007 Bluetongue Serotype 8 Epidemic Bulletin 15. http://www.efsa.europa.eu/etc/medialib/efsa/in_focus/bluetongue.Par.0041.File.dat/bluetongue_update_1_February_2007.pdf
33. **Losson B.**, (2007) Biting midges overwintering in Belgium. *Vet. Rec.* **160**, 451–452.
34. International Society for Infectious Diseases 2007a Bluetongue—Europe (14): BTV-8, Germany (Nordrhein-Westfalen), confirmed. ProMED-mail 2007; 13 June: 20070613.1928.
35. International Society for Infectious Diseases 2007b Bluetongue—Europe (41): BTV-8. Netherlands, Denmark.
36. International Society for Infectious Diseases 2007c Bluetongue—Europe (49): BTV-8. Switzerland.
37. World Organisation For Animal Health 2007d 29/11/2007: Bluetongue, Czech Republic (immediate notification). *Weekly Disease Information*, 20.
38. Defra. 2007 Press release—Bluetongue confirmed as circulating in East Anglia. <http://www.defra.gov.uk/news/2007/070922a.htm>

39. **Darpel K. E., Batten C. A., Veronesi E., Williamson S., Anderson P., Dennison M., Clifford S., Smith C., Philips L., Bidewell C., Bachanek-Bankowska K., Sanders A., Bin-Tarif A., Wilson A. J., Gubbins S., Mertens P. P., Oura C. A., Mellor P. S.,** (2009) Transplacental transmission of bluetongue virus 8 in cattle, UK. *Emerg Infect Dis.*, **15**, 2025-2028.
40. **Saegerman C., Berkvens D., Mellor P. S.,** (2005) Bluetongue epidemiology in the European Union. *Emerging Infectious Disease.* www.cdc.gov/eid. **14**.
41. **De Clercq K., Mertens P., De Leeuw I., Oura C., Houdart P., Potgieter A. C., Maan S., Hooyberghs J., Batten C., Vandemeulebroucke E., Wright I. M., Maan N., Riocreux F., Sanders A., Vanderstede Y., Nomikou K., Raemaekers M., Bin-Tarif A., Shaw A., Henstock M., Bréard E., Dubois E., Gastaldi-Thiéry C., Zientara S., Verheyden B., Vandenbussche F.,** (2009) Emergence of bluetongue serotypes in Europe, part 2: the occurrence of a BTV-11 strain in Belgium. *Transbound Emerg Dis.*, **56**, 355-361.
42. **Eschbaumer M., Hoffmann B., Moss A., Savini G., Leone A., Koing P., Zemake J., Conraths F., Beer M.,** (2010) Emergence of bluetongue virus serotype 6 in Europe--German field data and experimental infection of cattle. *Vet Microbiol.* **143**, 189-195.
43. **Meiswinkel R., Baldet T., de Deken R., Takken W., Delécolle J. C., Mellor P. S.,** (2008) The 2006 outbreak of bluetongue in northern Europe--the entomological perspective. *Prev Vet Med.*, **87**, 55-63.
44. **Trainer D. O, Jochim M. M.,** (1969) Serologic evidence of bluetongue in wild ruminants of North America. *Am J Vet Res.* **30**, 2007-11.
45. **Tessaro S. V, Clavijo A.,** (2001) Duration of bluetongue viremia in experimentally infected American bison. *J Wildl*, **37**, 722-9.
46. **Fischer-Tenhagen C., Hamblin C., Quandt S., Frölich K.,** (2000) Serosurvey for selected infectious disease agents in free-ranging black and white rhinoceros in Africa. *J Wildl*, **36**, 316-23.
47. **Linden A., Grégoire F., Nahayo A., Hanrez D., Mousset B., Massart A. L., De Leeuw I., Vandemeulebroucke E., Vandenbussche F., De Clercq K.,** (2010) Bluetongue Virus in Wild Deer, Belgium, 2005-2008. *Emerg Infect Dis.*, **16**, 83-86.

48. **Roy P.**, (1992). Bluetongue virus proteins. *J Gen Virol.*, **73**, 3051–64
49. **Verwoerd DW, Louw H, Oellermann R.A.**, (1970). Characterization of bluetongue virus ribonucleic acid. *J Virol*; **5**,1–7.
50. **Vewoerd, D.W.**, (1969). Purification and characterization of bluetongue virus. *Virology* **38**, 203-212.
51. **Roy P., Mark B., Noad R.**, (2009) Prospect for improved bluetongue vaccines. *Nature*, **7**, 120-128.
52. **Forzan, M., Marsh, M. & Roy, P.**, (2007). Bluetonguevirus entry into the cells. *J.virol*, **81**, 4819-4827.
53. **Zhang X, Boyce M, Bhattacharya B, Zhang X, Schein S, Roy P, Zhou ZH.**, (2010) Bluetongue virus coat protein VP2 contains sialic acid-binding domains, and VP5 resembles enveloped virus fusion proteins. *Proc Natl Acad Sci*, **107**, 6292-7.
54. **Forzan M, Wirblich C, Roy P.**, (2004) A capsid protein of nonenveloped Bluetongue virus exhibits membrane fusion activity. *Proc Natl Acad Sci*, **101**, 2100-5.
55. **Hewat, E. A., Booth, T. F., Loudon, P. T. & Roy, P.** (1992). Three-dimensional reconstruction of baculovirus expressed bluetongue virus core-like particles by cryo-electron microscopy. *Virology*, **189**, 10–20.
56. **Grimes, J. M. et al.** (1997). An atomic model of the outer layer of the bluetongue virus core derived from X-ray crystallography and electron cryomicroscopy. *Structure* **5**, 885–893.
57. **Burroughs, J. N., Grimes, J. M., Mertens, P. P. C. & Stuart, D. I.** (1995). Crystallization and preliminary X-ray analysis of the core particle of bluetongue virus. *J. Virology*, **210**, 217–220.
58. **Nason E.L., Rothagel R., Mukherjee S.K., Kar A.K., Forzan M., Prasad B.V.V., & Roy P.** (2004). Interaction between the Inner and Outer Capsid of Bluetongue Virus. *J. of Virology*, **78**. 8059-8067.
59. **Roy P.** (2008). Bluetongue virus: dissection of the polymerase complex. *J. of General Virology* **89**, 1789-1804.
60. **Roy P., Mark B. and Noad R.** (2009). Prospect for improved bluetongue vaccines. *Nature*, **7**, 120-128.
61. **Mertens P.P.C., Brown F. & Sangar D.V.**. Assignment of the genome segments of bluetongue virus type 1 to the proteins which they encode. *Virology*, **135**, 207-217.
62. **Roy P.**. Bluetongue virus genetics and genome structure. *Virus research*, **13**, 179-206.

63. **Hassan, S.S. & Roy, P.** (1999). Expression and functional characterization of bluetongue virus VP2 protein: role in cell entry. *J. Virol.* **73**, 9832-9842.
64. **Forzan, M., Marsh, M., & Roy, P.** (2007). Bluetongue virus entry into cells. *J. Virology*, **81**, 4819-4827.
65. **Hassan, S.H., Wirblich, C., Forzan, M. & Roy, P.** (2001). Expression and functional characterization of bluetongue virus VP5 protein: role in cellular permeabilization. *J. Virol.* **75**, 8356-8367.
66. **Roy P.**, (2008). Molecular Dissection of Bluetongue Virus, In: Thomas C. Mettenleiter and Francisco Sobrino. *Animal Viruses, Molecular Biology*. Norfolk, UK, Caister Academic Press. Chapter 7.
67. **Mellor P., Baylis M. & Mertens P.** (2009). Bluetongue. First edition, London, Academic Press.
68. **Mertens, P.P.C., Diprose, J.** (2004). The bluetongue virus core: a nano scale transcription machine. *J. Virus research*, **101**, 29-43.
69. **Fillmore, G.C., Lin, H. and Li, J.K.K.** (2002). Localization of the single-stranded RNA-binding domains of bluetongue virus nonstructural protein NS2. *J. Virol.* **76**, 499-506.
70. **Thomas, C.P. Booth, T.F., & Roy, P.** (1990). Synthesis of Bluetongue virus encoded phosphoprotein and formation of inclusion bodies by recombinant baculovirus in insect cells: it binds the single-stranded RNA species. *J. Gen. Virol.* **71**, 2073-2083.
71. **Lymperopoulos, K., Noad, R., Tosi, S., Nethisinghe, S., Brierley, I. & Roy, P.** (2006). Specific binding of bluetongue virus NS2 to different viral plus-strand RNAs. *J. Virology*, **353**, 17-26.
72. **Butan, C., Van Der Zandt, H. & Tucker, P. a.** (2004). Structure and assembly of the RNA binding domain of bluetongue virus non-structural protein 2. *J. Biol. Chem.* **279**, 37613-21.
73. **Taraporewala, Z.F., Chen, D., & Patton, J.T.** (2001). Multimers of the bluetongue virus nonstructural protein, NS2, possess nucleotidyl phosphatase activity: similarities between NS2 and Rotavirus NSP2. *Virology*, **280**, 221-31.
74. **Modrof, J., Lymperopoulos, K. & Roy, P.** (2005). Phosphorylation of bluetongue virus non-structural protein 2 is essential for formation of viral inclusion bodies. *J. Virol*, **79**, 10023-10031.
75. **Ross-Smith, N.** (2008). Elucidating the role of non-structural protein 2 (NS2) in bluetongue virus infection. PhD Thesis, University of Oxford.

76. **Parker, J.S., Broering, T.J., Kim, J. Higgins, D.E. & Nibert, M.L.**, (2002), Reovirus core protein mu2 determines the filamentous morphology of viral inclusion bodies by interacting with and stabilizing microtubules. *J. Virol.* **76**, 4483-4496.
77. **Ross-Smith, N.** (2008). Elucidating the role of non-structural protein 2 (ViP) in Bluetongue virus infection. PhD Thesis, University of Oxford.
78. **Fu, H.** (1995). Mechanisms controlling the infection of *Culicoides* biting mite with bluetongue virus. PhD Thesis, University of Hertfordshire, p. 154.
79. **Eaton, B.T., Hyatt, A.D., & Brookes, S.M.** (1990). The replication of Bluetongue virus. *J. Curr. Top. Microbiol. Immunol.* **162**, 89-118.
80. **Mertens, P.P.C., Maan, S. Samuel, A. & Attoui, H.** (2005) Orbivirus, Reoviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (eds), Virus Taxonomy, VIIIth Report of the ICTV. London, Elsevier/Academic Press, 446-483.
81. **French, T.J., & Roy, P.** (1990) Synthesis of bluetongue virus (BTV) assembly of double-shelled, virus-like particles of bluetongue virus by the simultaneous expression of four structural proteins. *J. Virol.* **64**, 5695-700.
82. **Limn, C.K., Roy, P.** (2003). Intermolecular interactions in a two-layered viral capsid that requires a complex symmetry mismatch. *J. Virol.* **77**, 1111-24.
83. **Karr, A.K., & Roy, P.**, (2003). Defining the structure-function relationships of bluetongue virus helicase protein VP6. *J. Virol.* **77**, 11347-56.
84. **Bhattacharya B., Noad R. J. and Roy P.** (2007). Interaction between Bluetongue virus outer capsid protein VP2 and vimentin is necessary for virus egress. *J. Virology*, **4**:7.
85. **Hyatt, A.D., Gould, A.R., Coupar, B. and Eaton, B.T.** (1991). Localization of the non structural protein NS3 in bluetongue virus-infected cells. *J. Gen. Virol.* **72**, 2263-2267
86. **Beaton, A.R., Rodriguez, J., Reddy, Y.K. & Roy, P.**, (2002). The membrane trafficking protein calpactin forms a complex with bluetongue virus protein NS3 and mediates virus release. *Proc. Natl. Acad. Sci. USA.* **99**, 13154-13159.
87. **Lee, J.W. & Roy, P.** (1986). Nucleotide sequence of a cDNA clone of RNA segment 10 of bluetongue virus (serotype 10). *J. Gen. Virol.* **67**, 2833-2837.
88. **Hyatt, A D., Zhao, Y. & Roy, P.** (1993). Release of Bluetongue virus-like particles from insect cells is mediated by BTV non-structural protein NS3/NS3A. *Virology*, **193**, 592-603.

89. **Guirakhoo, F., Catalen, J.A. & Monath, T.P.** (1995). Adaptation of bluetongue virus in mosquito cells results in overexpression of NS3 proteins and release of virus particles. *Arch. Virol.* **140**, 967-974.
90. **Raynal, P. & Pollard, H.B.** (1994). Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *Biochim. Biophys. Acta.* **1197**, 63-93.
91. **Hyatt, A.D., Gould, A.R., Coupar, B. and Eaton, B.T.** (1991). Localization of the non structural protein NS3 in bluetongue virus-infected cells. *J. Gen. Virol.* **72**, 2263-2267.
92. **Roy P.** (2008). Functional Mapping of Bluetongue Virus Proteins and Their Interactions with Host Proteins During Virus Replication. *Cell Biochem Biophys* **50**, 143–157.
93. **Huismans, H. & Els, H.J.** (1979). Characterization of the tubules associated with the replication of three different orbiviruses. *J. Virology.* **92**, 397-406.
94. **Barratt-Boyes, S.M. & MacLachlan, N.J.** (1995). Pathogenesis of bluetongue virus infection of cattle. *J. Am. Vet. Med. Assoc.* **206**, 1322-1329.
95. **Darpel KE, Monaghan P, Anthony SJ, Takamatsu H, Mertens PP** (2009) Bluetongue virus in the mammalian host and the induced immune response. In: Bluetongue, P Mellor, M Baylis and P Mertens, Eds, Elsevier, London, pp. 265-284.
96. **Mellor P., Baylis M. & Mertens P.** (2009). Bluetongue. *First edition, London, Academic Press.*, chapter 13, Clinical signs and pathology.
97. **Mellor P., Baylis M. & Mertens P.** (2009). Bluetongue. *First edition, London, Academic Press.*, chapter 12, Bluetongue virus in the mammalian host and the induced immune response.
98. **Schwartz-Cornil I, Mertens PP, Contreras V, Hemati B, Pascale F, Bréard E, Mellor PS, MacLachlan NJ, Zientara S.** (2008) Bluetongue virus: virology, pathogenesis and immunity. *Vet. Res.*, **39**, 46.
99. **Brewer AW, MacLachlan NJ** (1992) Ultrastructural characterization of the interaction of bluetongue virus with bovine erythrocytes in vitro. *Veterinary Pathology*, **29**, 356-359.
100. **MacLachlan, N.J.** (1994) The pathogenesis and immunology of bluetongue virus infection of ruminants. *Comparative Immunology, Microbiology and Infectious Disease*, **17**, 197-206.
101. **Gibbs E.P., Greiner E.C.**, (1994). The epidemiology of Bluetongue. *Comparative Immunology, Microbiology and Infection Disease*, **17**, 207-220.

102. **Maclachlan NJ, Osburn BI** (2008). Induced brain lesions in calves infected with bluetongue virus. *Veterinary Record*, **162**, 490e491.
103. **Toussaint, J.F., Sailleau C., Mast J., Houdart P., Czaplicki G. et al.** (2007) Bluetongue in Belgium, 2006. *Emerging Infectious Diseases*, **13**, 614-616.
104. **Dal Pozzo F, De Clercq K, Guyot H, Vanemeulebroucke E, Sarradin P et al.** (2009) Experimental reproduction of bluetongue virus serotype 8 clinical disease in calves. *Veterinary Microbiology*, **136**, 352-358.
105. **Anderson GA, Stott JL, Gershwin LJ, Osburn BI** (1987) Identification of bluetongue virus-specific immunoglobulin E in cattle. *J. of General Virology*, **68**, 2509-2514.
106. **DeMaula C.D., Leutenegger C.M., Jutila M.A., MacLachlan J.** (2002). Bluetongue virus-induced activation of primary bovine lung microvascular endothelial cells. *J.Veterinary immunology and immunopathology*, **86**, 147-157.
107. **DeMaula C.D., Leutenegger C.M., Bonneau K.R., & Maclachlan N.J.**(2002). The Role of Endothelial Cell-Derived Inflammatory and Vasoactive Mediators in the Pathogenesis of Bluetongue. *J. Virology*, **296**, 330-337.
108. **Mahrt, C.R. & Osburn, B.I.** (1986). Experimental bluetongue virus infection of sheep; effect of vaccination: pathologic, immunofluorescent, and ultrastructural studies. *Am. J. Vet.Res.* **47**, 1198-1203.
109. **Coetzer J. A. W., Thomson G. R., Tustin R. C.,** (1994) Infectious diseases of livestock with special reference to Southern Africa. *Oxford University Press* Section 4, Chapter 43, 443-459.
110. **Farina R., Scatozza F.,** (1995) Trattato di Malattie Infettive degli Animali. *Unione Tipografico-Editrice Torinese.*, Chapter 49, 844-849.
111. **Zhou, E.M., Afshar,** (1999). Characterisation of monoclonal antibodies to epizootic haemorrhagic disease virus of deer (EHDV) and bluetongue virus by immunization of mice with EDHV recombinant VP7 antigen. *Res.Vet. Sci*, **66**, 247-252.
112. **Zhou, E.M. & Chan, M:K.H.**(1996). Immunoblot assay as a confirmatory test of competitive ELISA for serodiagnosis of bluetongue. *J.Vet. Diagn. Invest.* **8**, 365-367.
113. **Martyn, J.C., A.R. Gould & Eaton B.T.** (1990). High level expression of the major core protein VP7 and the non-structural protein Ns3 of bluetongue virus in yeast :use of expressed VP7 as a diagnostic, group-reactive antigen in a blocking ELISA . *Virus Res.* **18**, 165-178

114. **Eaton, B.T., Gould A. R., Hyatt, A.D.Coupar, b.e.h., Martyn, J.C.& White J.R.** (1991). A BLUETONGUE serogroup-reactive epitope in the amino terminal half of the major core protein VP7 is accessible on the surface of bluetongue virus particles. *Virology* **180**, 687-696.
115. **French, T. & Roy, P.** (1990). Synthesis of bluetongue virus (BTV) corelike particles by recombinant baculovirus Expressing the two major structural proteins of BTV. *J. Virol*, **64**, 1530-1536.
116. **Mechan, J.O., & Wilson , W.C.** (2004) Antigen capture competitive enzyme-linked immunosorbent assay using baculovirus- expressed antigens for diagnosis of bluetongue virus and epizootic hemorrhagic disease virus. *J.Clin. Microbiol.* **42**, 518-523.
117. **Luo, L., & Sabara M.I.** (2005) Production of a recombinant VP7 major core surface antigen of epizootic haemorrhagic disease virus (EHDV) for use in serological diagnosis. *Clin. Diagn. Lab. Immunol.*, **12**, 904-909.
118. **Luckow, V. A., & Summers M.** (1988). Trends in the development of baculovirus expression vectors. *BioTechnology* **6**, 47-55.
119. **Schultz, G. & Delay, P. D.** (1955). Losses in newborn lambs associated with bluetongue vaccination of pregnancy ewes. *J. Am. Vet. Med. Assoc.* **127**, 224–226.
120. **Stott, J. L., Oberst, R. D., Channell, M. B. & Osburn, B. I.** (1987). Genome segment reassortment between two serotypes of bluetongue virus in a natural host. *J. Virol.* **61**, 2670–2674.
121. **Noad, R., Roy P.** (2009). Bluetongue vaccines. *Vaccine*, **27**, D86–D89
122. **Boone, J. D. et al.** (2007). Recombinant canarypox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep. *Vaccine*, **25**, 672–678.
123. **Roy P, French T, Erasmus B.J.** (1992). Protective efficacy of virus-like particles for bluetongue disease. *Vaccine*, **10**(1):28–32
124. **Perrin, A. et al.** (2007). Recombinant capripoxviruses expressing proteins of bluetongue virus: evaluation of immune responses and protection in small ruminants. *J. Vaccine.* **25**, 6774–
125. **Roy P., Bishop D.H., LeBlais H., Erasmus B.J.** (1994). Long-lasting protection of sheep against bluetongue challenge after vaccination with virus-like particles: evidence for homologous and partial heterologous protection. *J.Vaccine*, **12**, 805–811.

126. **Loudon PT, Hirasawa T, Oldfield S, Murphy M, Roy P.** (1991). Expression of the outer capsid protein VP5 of bluetongue viruses, and synthesis of chimeric double-shelled virus-like particles using combinations of recombinant baculoviruses. *Virology*, **182**, 793–780
127. **Sambrook J. & Russell D.W.** (2001). *Molecular Cloning: a laboratory manual*. Third edition by Cold Spring Harbor Laboratory Press, New York.
128. **Warming S., Costantino N., Court D. L., Jenkins N. A., Copeland N. G.,** (2005) Simple and highly BAC recombineering using galK selection. *Nucleic Acids Res.*, **24**, 33-36.
129. **Donofrio, G., S. Cavirani, A. Vanderplasschen, L. Gillet, and C. F. Flammini.** (2006). Recombinant bovine herpesvirus 4 (BoHV-4) expressing glycoprotein D of BoHV-1 is immunogenic and elicits serum-neutralizing antibodies against BoHV-1 in a rabbit model. *Clin Vaccine Immunol.*, **13**, 1246-1254.
130. **Donofrio, G., E. Bottarelli, C. Sandro, and C. F. Flammini.** (2006). Expression of bovine viral diarrhoea virus glycoprotein E2 as a soluble secreted form in a mammalian cell line. *Clin. Vaccine Immunol*, **13**, 698-701.
131. **Eaton, B. T., and A. D. Hyatt.** (1989). Association of bluetongue virus with the cytoskeleton. *Subcell Biochem* **15**, 233-273
132. **Eaton, B. T., A. D. Hyatt, and J. R. White.** (1987). Association of bluetongue virus with the cytoskeleton. *Virology* **157**, 107-116.
133. **Donofrio, G., C. Sartori, V. Franceschi, A. Capocéfalo, S. Cavirani, S. Taddei, and C. F. Flammini.** (2008). Double immunization strategy with a BoHV-4-vectorialized secreted chimeric peptide BVDV-E2/BoHV-1-gD. *Vaccine*, **26**, 6031-6042.
134. **Franceschi V., Capocéfalo A., Ravanetti L., Vanderplasschen A., Gillet L., Cavirani S., van Santen V. L., Donofrio G.,** (2010) Bovine herpesvirus 4 immediate early 2 (Rta) gene is an essential gene and is duplicated in bovine herpesvirus 4 isolate U. *Vet. Microbiol.*
135. **Donofrio G., Sartori C., Ravanetti L., Cavirani S., Gillet L., Vanderplasschen A., Flamini C. F.,** (2007) Establishment of a bovine herpesvirus 4 based vector expressing a secreted form of the bovine viral diarrhoea virus structural glycoprotein E2 for immunization purposes. *BMC Biotechnol.*, **18**, 7-68.