Expression Cloning of BPV-2 E6, E7 and L1 genes

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“An inefficient virus kills its host. A clever virus stays with it.”

James Lovelock

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Resumo

O Papilomavírus bovino tipo 2 (BPV-2) é um pequeno vírus de ADN, pertencente à família *Papillomaviridae* e ao género *Deltapapillomavirus*. O BPV-2 tem a capacidade de infetar os queratinócitos e fibroblastos, sendo capaz de causar fibropapilomas benignos no epitélio e na derme subjacente. A transformação dos fibroblastos sub-epiteliais pelo BPV-2 leva a uma acantose epitelial, e posteriormente a papilomatose.

O BPV, tal como outros Papilomavírus animais, é frequentemente usado como modelo de estudo, sendo importante para a clarificação dos mecanismos de patogénese, da interação entre o vírus, o hospedeiro e cofatores ambientais, assim como para a elucidação da biologia do vírus e da resposta imunitária desenvolvida pelo hospedeiro quando da infeção viral.

Este trabalho experimental foi realizado com o objetivo de expressar três genes do BPV-2 – os oncogenes E6 e E7, e o gene L1, através de uma metodologia de clonagem de expressão. Os genes E6 e E7 estão localizados na região inicial do genoma viral, estando relacionados com o processo de transformação das células hospedeiras. O gene L1 codifica a proteína estrutural mais representada na cápside viral. Devido à importância desta proteína na infecciosidade do vírus e na estimulação da resposta imunitária do hospedeiro, durante a infeção viral, o estudo da sua funcionalidade e a sua utilização como antigénio vacinal torna-a um alvo de estudo muito apelativo.

Palavras-chave: Papilomavírus Bovino tipo 2; Clonagem de Expressão; E6; E7; L1.

Abstract

The Bovine Papillomavirus type 2 (BPV-2) is a small DNA virus, belonging to the *Papillomaviridae* family and *Deltapapillomavirus* genus. BPV-2 infect both keratinocytes and fibroblasts, inducing benign fibropapillomas in the epithelium and in the underlying dermis. The transformation of sub-epithelial fibroblasts by BPV-2 leads to epithelial acanthosis, and papillomatosis.

Animal papillomavirus models are frequently studied in order to understand PV's life cycle and pathogenesis. BPV is one of the animal PV's studied to provide information regarding PV pathogenesis and biology, and also the interaction between the virus and the host and environmental cofactors, and the host's immunological response to viral infection.

The aim of this study was to express three BPV-2 genes – the E6 and E7 oncogenes and the L1 gene, by expression cloning methodology. The E6 and E7 genes are located in the 5' region of the BPV-2 genome, and are related to the virus transforming activity. The L1 gene codes the major structural protein of the viral capsid. Due to L1 importance in the virus infectivity and in the host immunological response, the study of its use as a vaccine candidate, makes it an important and relevant target to investigate.

Keywords: Bovine Papillomavirus type 2; Expression Cloning; E6; E7; L1.

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List of Abbreviations

ABTS	**2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)**
ATP	**Adenosine Triphosphate**
BPV	**Bovine Papillomavirus**
BSA	**Bovine serum albumin**
CEH	**Chronic Enzootic Hematuria**
DNA	**Deoxyribonucleic acid**
DPV	**Deer Papillomavirus**
E. coli	***Escherichia coli***
EEPV	**European elk Papillomavirus**
ELISA	**Enzyme-linked immunosorbent assay**
HPV	**Human Papillomavirus**
HSPG	**Heparin Sulfate Proteoglycan**
IgG	**Immunoglobulin G**
IPTG	**Isopropyl β-D-1-thiogalactopyranoside**
LB	**Luria Bertani Broth**
LCR	**Long Control Region**
mRNA	**Messenger RNA**
NLS	**Nuclear Localization Signals**
ORF	**Open Reading Frame**
OvPV	**Ovine Papillomavirus**
PAGE	**Polyacrylamide Gel Electrophoresis**
PBMC	**Peripheral Blood Mononuclear Cell**
PBS	**Phosphate-buffered saline**
PCR	**Polymerase Chain Reaction**
PDGF	**Platelet-Derived Growth Factor**
PV	**Papillomavirus**
PVDF	**Polyvinylidene difluoride**
RNA	**Ribonucleic Acid**
RPM	**Rotations per minute**
SDS	**Sodium dodecyl sulfate**
SOC	**Super Optimal broth**
TBS	**Tris-buffered saline**

URR	**Upstream Regulatory Region**
VLP	**Virus-Like Particle**
MW	**Molecular Weight**

Chapter 1

INTRODUCTION

1.1 Papillomaviruses

Papillomaviruses (PVs) are a group of small DNA viruses that usually cause benign infections in vertebrates. They infect the epithelia of their hosts, where they can persist asymptomatically, being found in numerous mammal, bird and reptile species, suggesting a papillomavirus-host coevolutionary history. Although currently Papillomaviruses belong to the *Papillomaviridae* family, originally they were included in the *Papovaviridae* family due to morphological and DNA similarities with papillomaviruses (Antonsson & Hansson, 2002; Bernard et al., 2009; Chan et al., 1992; Van Doorslaer, 2013).

Within the *Papillomaviridae* family, 29 genera are currently considered and identified according to the Greek alphabet, *e.g.*, *Deltapapillomavirus*. The inclusion of the different species inside the several genera is based on phylogenetic analysis, biological properties and nucleotide sequence similarity of the L1 gene ORF (open reading frame), due to its high conservational pattern within each taxonomic genus (Chan et al., 1992; de Villiers, Fauquet, Broker, Bernard, & zur Hausen, 2004). The PV species include different viral types, and are named according to the best representative viral type; the remaining viral types are designated viral strains. The viral types are identified according to the taxonomic name of the natural host, with the exception of *Bos taurus* Papillomaviruses, usually named as BPV (Bovine Papillomaviruses) (de Villiers et al., 2004; Lunardi et al., 2013).

The successful amplification and sequencing of PVs full genomes was an important achievement towards their taxonomic classification, with HPV-1, HPV-16 and BPV-1 the first reported genomes. Presently, almost all PV genomes have been effectively sequenced (de Villiers et al., 2004); by the analysis of the nucleotide sequences and their biological and pathological characteristics, it is possible to classify PVs isolates by genus, specie and viral type. When two PV isolates exhibit less than 60% similarity in their L1 ORFs and 23% to 43% similarity on their full genomic sequences, they are included in different genera. Within the same genus, different species present 60% to 70% similarity of their L1 ORFs. Finally, when two isolates exhibit 71 to 89% similarity in their L1 ORFs, they are considered different viral strains of a specie (Lunardi et al., 2013).

The Bovine papillomavirus type 2 (BPV-2) is included in the *Deltapapillomavirus* genus of the *Papillomaviridae* family, together with Bovine Papillomavirus type 1 (BPV-1), European elk papillomavirus (EEPV), Deer papillomavirus (DPV) and Ovine papillomavirus type 1 (OvPV-1) (Bernard et al., 2009; Lunardi et al., 2013).

BPV-1 and BPV-2 are associated with the development of benign tumors in the epithelium and underlying derma (fibropapillomas) of the host (G Borzacchiello, 2007). BPV-1 and BPV-2 are closely related genotypes, sharing 92% homology in their L1 protein sequence (Hainisch, Brandt, Shafti-Keramat, Van den Hoven, & Kirnbauer, 2012).

One of the most important methods used for the understanding of PVs life cycle and pathogenesis was the study of animal PVs models. Among other animal PVs, BPV is used as a model to understand the PV pathogenesis, including the interaction between the virus and its host, the environmental cofactors, and also the host's immune response to the virus. Furthermore, BPV was of major importance in the

development of vaccines against PVs (Giuseppe Borzacchiello, Roperto, Nasir, & Campo, 2009; M Saveria Campo, 2006). Farm animals, mainly cattle, can be affected by BPV-induced lesions, which leads to production losses; hence, the research on this virus is important to minimize the virus pathogenic impact in the agricultural sector (M Saveria Campo, 2006; Cota, Peleteiro, Petti, Tavares, & Duarte, 2015).

1.2 Bovine Papillomavirus Virion

The BPV virion presents a non-enveloped structure, with an icosahedral capsid of 55-60nm diameter, constituted by the major L1 and minor L2 structural proteins. Through an atomic model already generated, it is known that a part of the C-terminus of L1 protein is exposed on the virus surface, suggesting that it might have a role in infection and immunogenicity (M Saveria Campo, 2006; Modis, Trus, & Harrison, 2002).

The L1 structural protein is arranged in the viral capsid in 72 pentamers associated in a T=7 icosahedral symmetry (Roden, Kirnbauer, Jenson, Lowy, & Schiller, 1994). Inside this structure, BPV virion exhibits a double-stranded circular DNA genome, condensed by the action of cellular histones (Giuseppe Borzacchiello & Roperto, 2008; Modis et al., 2002).

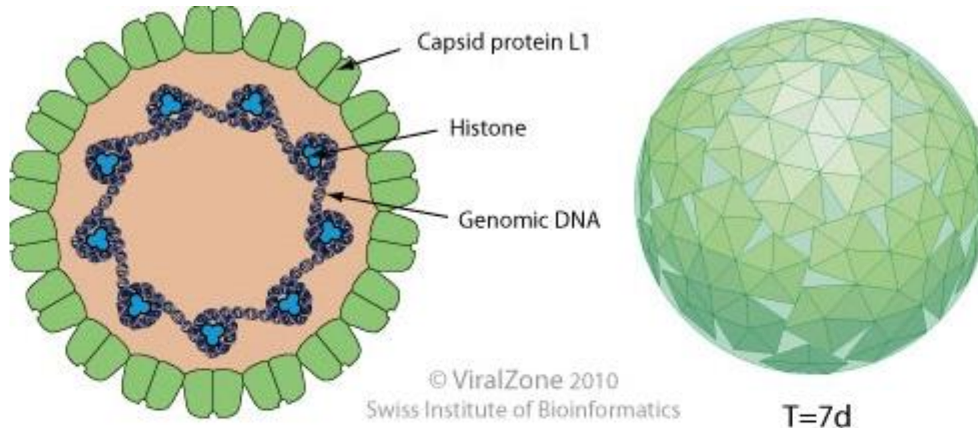

Figure 1 – Schematic representation of Papillomaviruses virion. Extracted from (Viral Zone, 2010).

1.3 Bovine Papillomavirus type 2 Genome

The BPV-2 genome has approximately 8Kbp and is divided in three regions: the Long Control Region (LCR) or Upstream Regulatory Region (URR), a non-coding sequence including the essential elements for viral DNA replication and transcription; the remaining two regions are identified as early (E) and late (L) genes according to the host's cell differentiation stage where they are expressed (Lunardi et al.,

2013). The early genes code six non-structural proteins (E1, E2, E4, E5, E6 and E7) necessary for the replication and transcription of the virus genome, and are expressed in the undifferentiated or intermediately differentiated keratinocytes. The late genes encode the two capsid proteins (L1 and L2), and are expressed in terminally differentiated keratinocytes (Giuseppe Borzacchiello & Roperto, 2008; M Saveria Campo, 2006; Lunardi et al., 2013; Zheng & Baker, 2006). One of the characteristics of some PV is their polycistronic genomes, where a polypeptide can be coded from an ORF that spans different separated exons, and one mRNA sequence has the information for the synthesis of more than one protein. Two examples of the polycistronic genome of BPV-2 are the E1^E4 and E8^E2 ORFs.

The three genomic regions (early region – late region – long control region) are separated by two polyadenylation sites (pA), the early pA and late pA (Zheng & Baker, 2006).

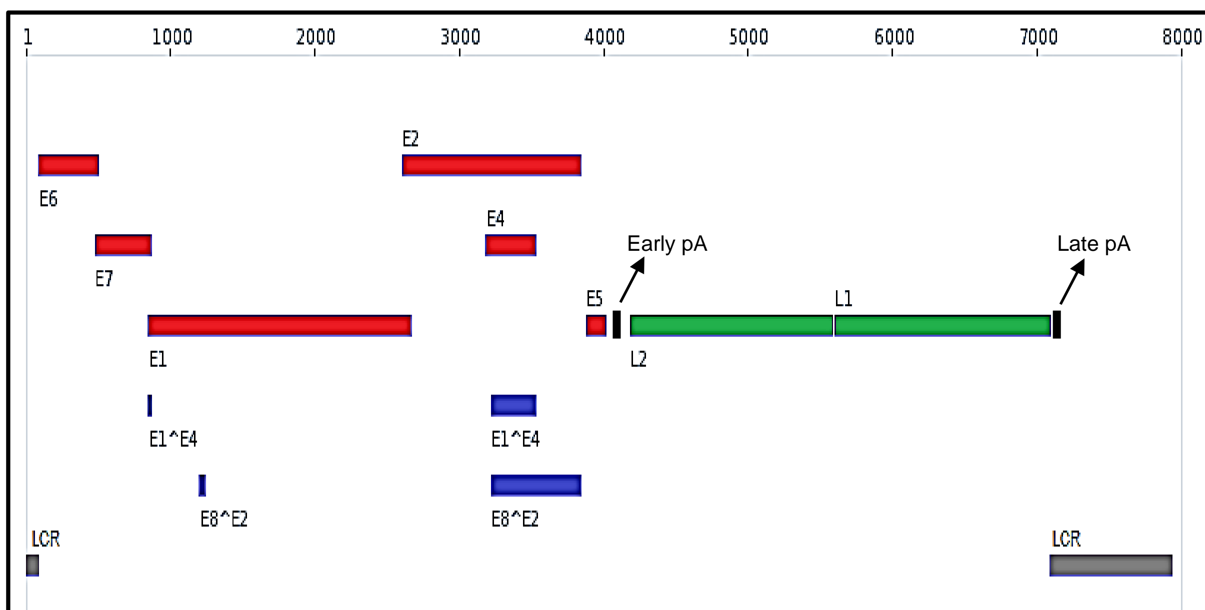

Figure 2 – Linear representation of the circular BPV-2 genome, in a bp scale. Adapted from (NIAID Office of Cyber Infrastructure and Computational Biology, 2012).

1.3.1 BPV Long Control Region (LCR)

As mentioned before, the long control region (LCR) of BPV-2 is a non-coding sequence that includes the regulatory elements necessary for the replication and transcription of the viral genome. This region is positioned before the 5'-end of the early region and after the 3'-end of the late region, and has approximately 500-1000 nucleotides (Giuseppe Borzacchiello & Roperto, 2008). These regulatory elements have a crucial role on the biology of the virus, determining the type of epithelial cell the virus can infect, controlling the early and late genes expression, and adjusting the virus biology when in the presence of hormones and growth factors. Regulatory proteins coded by the early genes (*e.g.*, E2 protein), recognize and bind to the regulatory elements, where can function as transcriptional activators, repressors, terminators or replication initiators (Bernard, 2013). The origin of replication, that is present

in the BPVs LCR, is composed by three elements: At least one E2 protein binding site, an A+T rich region and an E1 protein binding site (McBride, 2013; Ustav, Ustav, Szymanski, & Stenlund, 1993).

In the LCR of BPV-2 genome twelve E2 binding sites are present, and by the interaction of the E2 protein to these sites, the viral genes transcription is controlled, being activated or repressed (Giuseppe Borzacchiello & Roperto, 2008). The E1 protein recognize the origin of replication in the LCR, which is important for the virus DNA replication (Lunardi et al., 2013).

1.3.2 BPV Early Region (E)

The E1 ORF is the largest encountered in the early region, and the proteins that result from this gene expression in several PVs, present a significant amino acidic similarity, being highly conserved. The protein encoded by this gene – E1 protein – is an enzyme, an ATP-dependent DNA helicase, and together with E2 protein, is extremely important for the virus DNA replication, recognizing the origin of replication (Bergvall, Melendy, & Archambault, 2013; Lunardi et al., 2013). The E1 protein has a role in the increase of the viral episomes after basal keratinocytes infection and in the conservation of the viral episome levels in the epithelial cells that initiate the differentiation stage. The bi-directional replication of the viral genome, which occurs in the uppermost differentiated layers of the epithelium, is also dependent and promoted by the E1 protein. After the recognition of the origin of replication, localized in the LCR, the E1 protein adopts its enzymatically active form, as a double-hexameric helicase, with the ability to unwind the origin of replication and the DNA in front of the replication fork. The E1 protein can be divided in three different segments, according to their functional roles: an N-terminal regulatory region that is crucial for the optimal replication of the viral genome, a DNA-binding domain that recognizes particular sites in the origin of replication, and a C-terminal enzymatic domain that is responsible for the unwinding of the viral DNA and has an ATPase activity. The viral genome replication is also dependent of the cellular replication machinery activity, and the E1 protein plays a major role in its recruitment (Bergvall et al., 2013).

The E1 protein is a high-value model for helicase activity studies, allowing the enlightening of the mechanisms of DNA replication in eukaryotes (Bergvall et al., 2013).

The E2 protein has an important role in the transcription of the viral genes and in the maintenance of the viral genome on its episomal form. This protein is also important for the segregation of the viral genome to daughter cells during cell division, guaranteeing an equal distribution of the DNA of the virus, by supporting binding between these episomes and mitotic chromosomes. This protein also participates in the viral genome replication, binding to the origin of replication in cooperation with the E1 protein, promoting the recruitment of the double-hexameric helicase E1 (Giuseppe Borzacchiello & Roperto, 2008; Lunardi et al., 2013). One shorter form of this protein can be expressed – an E2 fusion protein – formed by the link between sequences of an alternative and specific ORF of the E1 region – E8 – and the C-terminus of E2. This peptide is translated by the splicing of E8 and E2 exons. The alternative ORF

of the E1 region of the genome that is necessary for the synthesis of this E2 fusion protein is called E8, and the final fusion protein is designated E8^E2 protein. The E8^E2 protein acts as a viral genome transcription and replication repressor, due to competition for the viral genome binding sites with the protein E2 and by the recruitment of repressing complexes (McBride, 2013).

The nature of the viral genome binding sites where the E2 protein binds and of the recruited cellular factors, promote the repression or activation of the transcriptional process (McBride, 2013). Some studies performed with BPV-1 have suggested that the E2 protein can also participate in the viral genome packaging into viral particles (McBride, 2013; Zhao et al., 2000).

Although the early genes are expressed in the early or intermediate stages of epithelial cells maturation (undifferentiated or intermediately differentiated keratinocytes), the E4 protein is mainly translated in the terminally differentiated keratinocytes. E4 is abundantly present in the cytoplasm of these cells, supporting the productive phase of the viral DNA replication (Giuseppe Borzacchiello & Roperto, 2008; Lunardi et al., 2013). The E4 ORF is inserted within the E2 ORF, as an example of the polycistronic character of the PV genomes. The E1^E4 protein is another fusion protein translated by a spliced mRNA that includes sequences of the E1 ORF (Doorbar, 2013).

The E5 protein belongs to the viral oncoproteins group, together with the E6 and E7 proteins, and is the major transforming oncoprotein in BPV-2. This group of oncoproteins have the ability to stimulate cell proliferation and survival, and modulate the differentiation of keratinocytes (Vande Pol & Klingelhutz, 2013).

E5 is a small and hydrophobic transmembrane polypeptide with an alfa-helical configuration. With 44-amino acid length, it can be divided in two domains: a mainly hydrophobic amino-terminal domain, and a small hydrophilic carboxyl-terminal domain. The BPV E5 oncoprotein is expressed in the cytoplasm of the host basal and suprabasal epithelial cells, mainly localized in the membranes of the Golgi apparatus and Endoplasmic Reticulum. In cell cultures, when this protein is overexpressed, it can be found in the plasma membrane. The cells transformation by this oncoprotein results from the activation of kinases, not from an E5 intrinsic enzymatic activity (G Borzacchiello, 2007; Lunardi et al., 2013; Sante Roperto et al., 2014; Venuti et al., 2011). In BPV, the E5 protein binds to the platelet-derived growth factor β receptor (PDGF β receptor), activating this transmembrane tyrosine kinase (G Borzacchiello et al., 2006; Daniel DiMaio, Lai, & Mattoon, 2000; C. C. Lai, Henningson, & DiMaio, 1998; C.-C. Lai, Edwards, & DiMaio, 2005). The ligation between the E5 protein and the PDGF β receptor results in the dimerization and *trans*-phosphorylation of the receptor, in a ligand-independent way. By this ligation, transformation, cellular signaling and cell proliferation are induced. Additionally, the E5 protein can bind to a transmembrane subunit of the vacuolar H+-ATPase proton pump, causing its impairment, which can result in a pH increase of the Golgi apparatus, endosomes and cytosol (D DiMaio & Mattoon, 2001). The alkalinization of the Golgi apparatus causes a down-regulation of the Major Histocompatibility Complex class I expression, inhibiting its export to the cell surface, hiding the BPV infection from the host's immune-response (G Borzacchiello et al., 2006; Giuseppe Borzacchiello & Roperto, 2008; Daniel DiMaio & Petti, 2013; C.-C. Lai et al., 2005; Marchetti, Ashrafi, Tsirimonaki, O'Brien, & Campo, 2002;

Sante Roperto et al., 2014). Another consequence of the pH increase within the cell organelles can be a loss of cell-to-cell communication. In this way, the transformed cells can lose sensibility to homeostatic growth control signals from the surrounding normal cells (Venuti et al., 2011).

The E6 protein is important in cell transformation and immortalization, although some BPV types lack the E6 oncogene (BPV-3, BPV-4 and BPV-5) (Corteggio, Altamura, Roperto, & Borzacchiello, 2013). The endogenous expression of the E6 protein has been highly difficult to study, due to the low expression levels of the protein and the unavailability of sensitive antibodies (Howie, Katzenellenbogen, & Galloway, 2009). The E6 protein has 137 amino acids length, and is constituted by two zinc-bind domains, allowing an interaction of the protein with the DNA. In BPV-1 and BPV-2, the E6 protein can function as a transcriptional activator, interacting with the focal adhesion protein paxillin and the cell-cycle regulator p53, and it has also a telomerase activity (Mazzuchelli-de-Souza et al., 2013; Wade, Brimer, & Vande Pol, 2008). Paxillin is a protein that is inserted in a transducing signals mechanism from the plasma membrane to focal adhesions and the actin cytoskeleton (Tong & Howley, 1997). In BPV-1, changes of the actin cytoskeleton by disruption of the actin fiber formation in E6-transformed cells was already confirmed, due the interaction of the E6 protein with the protein paxillin (Mazzuchelli-de-Souza et al., 2013; Tong & Howley, 1997). Cell morphology, cell motility, cell division, cell-to-cell contact and the contact between cells and the extracellular matrix are dependent of the actin cytoskeleton. The action of E6 in the disruption of the actin fiber formation affects all these actin cytoskeleton-depending processes. In this way, the E6 protein has an important interference role in cellular signal transduction and cell-cycle of transformed cells. Furthermore, the anchorage-independent growth of the BPV-1 transformed cells is a phenomenon that requires the activity of the E6 protein (Tong & Howley, 1997). Anchorage dependence is necessary for regulated cell growth, cell proliferation, migration and cell differentiation in non-tumorigenic cells (DeMasi, Chao, Kumar, & Howley, 2007).

Finally, the binding of the E6 protein to paxillin is correlated with the transforming competence, suggesting an important role of the E6/paxillin interaction in the oncogenic process (Tong & Howley, 1997).

The E6 oncoprotein can also interact with p53 cell-cycle regulator, a tumor suppressor and transcription factor. This tumor suppressor protein is present in the cell in low levels and transcriptionally inactive. The p53 levels increase and it is activated when occurs cellular damage and, based on the type and extent of the cellular damage, p53 can have a role in DNA repair pathways initiation, cell cycle arrest and apoptosis (Howie et al., 2009; Li, Zhao, Liu, Han, & Fan, 2005). In BPV-1, the interaction of E6 protein with p53 downregulates its transcriptional activity, but do not degrades it. The E6 protein inhibits the function of the CBP/p300 transcriptional coactivator, important for the transcriptional activity of p53 (Zimmermann et al., 2000).

The vesicular trafficking pathway is another target for the BPV-1 E6 protein. The biosynthesis and delivery of cell proteins engaged in this pathway, as the major histocompatibility complex class II proteins, are affected by the E6 action over the vesicular trafficking pathway (Tong, Boll, Kirchhausen, & Howley, 1998).

The E7 protein is also included in the oncoproteins group, together with E5 and E6. It is a zinc-binding protein, constituted by 127 amino acids, with transforming activity, although few is known about its involvement in carcinogenesis (G Borzacchiello, Resendes, Roperto, & Roperto, 2009). The E7 oncoprotein can bind to p600, a protein that has an important role in cell survival and morphogenesis, being hypothesized that it may play a role in apoptotic signaling, protecting the cells from apoptosis, leading to better conditions of anchorage-independent growth. It has been suggested that the binding to p600 is correlated with the BPV-1 E7 protein transforming activity. Furthermore, as already documented, the BPV-1 E7 protein can't by itself induce anchorage independence, but its activity has the potential to enhance the transformation activity of E5 and E6 (Corteggio, Di Geronimo, Roperto, Roperto, & Borzacchiello, 2011; DeMasi et al., 2007; DeMasi, Huh, Nakatani, Münger, & Howley, 2005).

This oncoprotein has also the potential to inhibit the cell detachment-induced apoptosis, named as anoikis. Anoikis is frequently inhibited in cancer cells in order to enable their survival, when there is an absence of normal cell-matrix interactions. Some studies have already demonstrated that the ability of E7 to inhibit anoikis is associated in part with the ability that this protein has to interact with p600. These studies have also showed that the E6 oncoprotein have a role in anoikis resistance, although at different life cycle stages of the virus (DeMasi et al., 2007).

Some studies using imunnolocalization have demonstrated that in fibropapillomas caused by BPV-1 and BPV-2, the E5 and E7 proteins are present in similar cellular areas, being both proteins mostly expressed in the cytoplasm of the host's basal epithelial cells. Some nuclear expression was also detected. The coexpression of E5 and E7 in basal cell layer may suggest that these proteins have a cooperative role in cell proliferation control and that E7 expression may be important to modulate the E5 actions on epithelial cells.

1.3.3 BPV Late Region (L)

The late region of PV genome is constituted by the L1 and L2 genes, coding the L1 and L2 structural proteins that are present in the viral capsid, playing crucial roles in the virion assembly and on the infectious process (Lunardi et al., 2013; Wang & Roden, 2013a). The L1 protein is the major capsid protein and represents about 80% of the total PV protein (G Borzacchiello, 2007; Favre, 1975). The epithelium differentiation triggers the L1 and L2 genes expression and viral genome replication increase, leading to the assembly of viral particles in the nucleus (Day, Roden, Lowy, & Schiller, 1998).

The two structural proteins of the virus are synthetized in the cytoplasm, and then recruited to the nucleus, due to the presence of nuclear localization signals (NLS) in these proteins. Nuclear localization signals are small stretches of amino acids that are mainly constituted by lysine and arginine. Once in the nucleus, the assembly of PV virons occurs (Paintsil, Müller, Picken, Gissmann, & Zhou, 1996). It is suggested that the L2 protein has the capacity to recruit the major capsid L1 protein and an E2-viral genome complex to specific nuclear interchromatinic substructures, known as Promonocytic Leukemia

Protein Oncogenic Domains (POD's). The co-localization of L2, L1 and E2-viral genome complex at POD's is essential for PV assembly (Day et al., 1998).

The L1 protein has the ability to self-assemble in the absence of L2, in a spontaneously way, into virus-like particles (VLPs). The VLPs are non-infectious empty-capsid structures, highly similar to the PV virion, without PV genome (Buck, Day, & Trus, 2013; Kirnbauer, Booy, Cheng, Lowy, & Schiller, 1992). VLPs are also antigenically highly similar to PV virions, having the ability to induce high titers of type-specific neutralizing antibodies (Hainisch et al., 2012).

The exterior surface of the virus is mainly constituted by the L1 protein, and therefore, it must have an important role in the attachment of the virus to the host cell receptor. After the attachment to the host cells, L1 must have the capability to become more flexible in order to release the viral genome into the cell (Buck et al., 2013). Studies suggest that the binding between L1 and host receptors induces conformational changes in the virus capsid. Consequently, the L2 protein is exposed in the viral capsid, posteriorly participating in the host cell entry process (Wang & Roden, 2013a). However, the study of the infection mechanisms, especially the investigation of putative PV receptors, have been hampered by the difficulty to generate PVs in vitro (Evander et al., 1997; Roden et al., 1994).

The virus binding to the host cells membrane is possible by the interaction between the L1 protein and heparin sulphate carbohydrates present on proteoglycans (HSPG), and this seems to be necessary for a successful infection (Giuseppe Borzacchiello & Roperto, 2008; Buck et al., 2013).

Roden et al. suggested that PVs bind to an evolutionary conserved receptor, expressed on several cell types. The wide host range of BPV is consistent with this suggestion. The interaction between PV virions and α6 β4 integrin suggest that it may be a cellular receptor for PVs (Evander et al., 1997; McMillan, Payne, Frazer, & Evander, 1999). Integrins are heterodimeric glycoproteins involved in cell-to-cell and cell-matrix interactions (Evander et al., 1997).

In BPV-1, the virion entry in host cells occurs through a clathrin-dependent endocytosis process. After ligation to the host receptors, clathrin-coated pits are formed. After entering the host cell, the BPV-1 virion uses the caveolar pathway for infection (Laniosz, Holthusen, & Meneses, 2008). Afterwards, the virion is transported to early and late endosomes, and due to the low pH registered inside these endosomal compartments, the viral genomic DNA is exposed. After the trafficking of the virion to early and late endosomes, the involvement of L1 in the entry process terminates (Buck et al., 2013).

The domains and function of L2 appears to be strongly conserved in all PV types, and animal PVs models are extensively used for the study of the L2 protein. Unlike L1, the L2 protein does not self-assemble and do not form VLPs by its own, but when co-expressed with L1 it can integrate the L1-formed VLP. The BPV-1 L2 protein has L1-binding sites allowing a close interaction between the two proteins. In the capsid of the virion, L2 is predominantly hidden below the surface, and the interaction of the capsid with HSPG and the L2 cleavage by furin causes a conformation change, responsible for the exposure of a L2 portion. In parallel to L1, this minor capsid protein has also a DNA-binding activity, required for its role in viral encapsidation (Wang & Roden, 2013a). Another characteristic of L2, linked to its role in the viral genome encapsidation, is its ability to bring components of the papillomavirus virion

to specific locals of the nucleus, where the virus assembly occurs. Through the L2 protein action, the E2, L1 and the viral genome are co-localized in this specific location of the nucleus, facilitating the viral genome encapsidation and virion assembly (Day et al., 1998; Wang & Roden, 2013a).

As stated above, L2 also participates in the infectious process through its interaction with a secondary receptor. Some authors suggest that L2 exposure, during the conformational changes in the virion capsid, allows it's interaction with a secondary receptor, consequently conducting to the internalization of the virion. However, the role of L2 in binding and interaction with a secondary receptor of the host cells is a topic that remains controversial, still deserving debate and research (Wang & Roden, 2013a).

After entering the host cells, the PV virion must cross the cytoplasm in order to the viral genome reach the nucleus. The L2 protein has an important role in this trafficking process. Studies made in HPV have proposed that L2 forms a complex with the viral genome, a crucial event for the viral genome transport to the nucleus; it may also use the cytoskeleton in order to transport the viral genome to the nucleus. Therefore, it is clear that L2 participates in several processes relative to the virion assembly and virus infection (Wang & Roden, 2013a).

1.4 PV Life Cycle

The PVs life cycle is closely related with the differentiation process of the host epidermis. The differentiation stages of the infected basal cells towards the epithelium surface is important for the regulation of the viral genes expression (Doorbar, 2005). The stratified epithelium of the cutaneous or mucosal epithelium of the epidermis is composed by several layers, and plays an important role as a barrier against pathogen invasion and water loss. Stem cells constitute the basal layer of the stratified epithelium and they continuously divide to replenish the layers above, in order to maintain the tissue integrity. The keratinocytes – the predominant cell type in epidermis – undergo a sequence of morphological and biochemical changes, resulting in the formation of several cellular layers of the stratified epithelium of epidermis. From the bottom to the top, the stratified epithelium is composed by the: *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum*. The dead cornified cells, at the top of these epithelial layers, are frequently sloughed into the environment (Jia & Zheng, 2009; Sakakibara, Chen, & McBride, 2013).

After infecting the *stratum basale* cells, the virus maintains its genome as a low-copy replicating plasmid and its genes expression at a low rate, just enough to allow the replication and the maintenance of the viral genome. This viral profile allows cell proliferation without alerting the host immune response. The late viral gene expression and genome amplification are then induced and initiated when the infected cells start their differentiation process (Sakakibara et al., 2013). In the spinous layer, the virus-infected cells reenter the cell cycle, which results in the amplification of the viral genome to high copies, necessary for the virus production (Wade et al., 2008).

Hence, the high-level viral gene expression and replication are limited to the most differentiated cells, where these processes are invisible to the host's immune system (Sakakibara et al., 2013).

The PVs life cycle is divided in three different phases of genome replication. The first phase occurs when the virus infects the keratinocytes present in the *stratum basale*. In this phase, the virus replicates its genome in an unlicensed way, in order to synthetize a small amount of viral genomes. The second phase occurs when the basal cells are in the dividing process, and when the host DNA replicates and is segregated to daughter cells. In this second phase, the viral genomes replicate in accordance with the host DNA genome replication. At this moment, the daughter cells can either proceed to the differentiating pathway, or stay in the basal layer where they continue the division process. The cells that follow the differentiating pathway will move to the top layers of the epithelium, inducing the third phase of viral genome replication. However, the infected basal cells can be maintained as a reservoir of infected cells, which is important to the continuous flux of infected cells that differentiate and posteriorly produce viral particles. In the third phase of the viral genome replication, the late genes expression is activated, occurring the vegetative viral genome amplification. The viral genomes are synthetized at large numbers in this phase, being posteriorly packaged in viral capsids (Sakakibara et al., 2013).

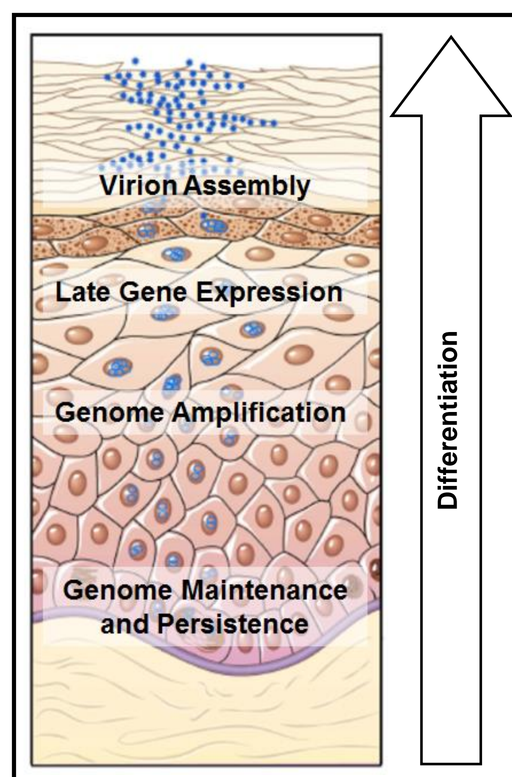

Figure 3 – Schematic representation of the relation between the papillomavirus life cycle and the host's epithelium differentiation. Adapted from (Sakakibara et al., 2013).

One characteristic of Papillomaviruses is their ability to establish latent infections (Maglennon & Doorbar, 2012). The BPV viral genome has been already detected in normal epithelia without any

apparent papilloma, suggesting a latent or sub-clinical infection (Ogawa et al., 2004). Commonly, at site of trauma, reactivation of BPV may occur. This event suggests that a latent form of BPV DNA is present in these sites, and the damage of the epithelium leads to the formation of a papilloma, by inducing the viral genes expression. The BPV DNA has been detected in the iliac lymph nodes, urine, milk and seminal cells (Cota et al., 2015; Lindsey et al., 2009). Furthermore, the BPV DNA can also be detected in the circulating lymphocytes of cattle, in an episomal form, suggesting that the epithelium may not be the only site where the virus can be present in a latent form (M Saveria Campo, 2006). It was proposed that reactivation from latency would occur and accompany immunosuppression. Hereupon, alteration in the immune status would affect the viral copy-number, and stimulate the appearance of visible papillomas (Maglennon & Doorbar, 2012). Additionally, damage of the epithelium induces viral genes expression through the production of inflammatory cytokines and by cell proliferation stimulation, leading to the formation of papilloma (M S Campo, 2003).

1.5 BPV-2 Pathogenesis

BPV-1 and BPV-2 are the only known PVs that present a cross-species infection, infecting several equids (M Saveria Campo, 2006). BPV-2, along with BPV-1, is related to the appearance and progression of skin non-metastasizing tumors – sarcoids – in horses, donkeys and mules, the most common neoplasm in these animals (Hainisch et al., 2012). In equids that suffer from sarcoids, no virions can be detected, suggesting a viral abortive infection. Furthermore, studies have detected DNA from the BPV E5 ORF and E5 RNA transcripts in equine sarcoids, which strongly suggests the involvement of the virus in the sarcoid development (M S Campo, 2003; Chambers et al., 2003; van Dyk et al., 2009). Furthermore, it was also demonstrated that BPV-2 plays an important role in bubaline bladder carcinogenesis (Maiolino et al., 2013).

These two BPV types have also the ability to infect both epithelial and mesenchymal cells. The transformation of sub-epithelial fibroblasts by these BPV types, leads to epithelial acanthosis, and papillomatosis – a skin surface elevation or projection, known as cutaneous or skin warts. The epithelial lesion caused by BPV infection, usually regress spontaneously, in immunocompetent animals, without serious clinical complications for the host (M S Campo, 2003; Sante Roperto et al., 2013). BPV-2 can also infect the urinary bladder epithelium and esophagus, wherein no virions are produced. It had been suggested that this virus is transmitted by direct contact between animals, through open skin growths and fomites that already have been in contact with infected animals. Some insects may be vectors through which the virus may be transmitted (Samour, 2012).

The concomitant ingestion of immunosuppressants by the BPV-2 infected animal can lead to the development of malignant carcinomas in the urinary bladder (Hainisch et al., 2012). In the skin and esophagus, only benign proliferation are caused (M S Campo, 1988). In the urinary bladder, the progression to malignant carcinomas is not accompanied by viral maturation, i.e., by virion production (M S Campo, 2003).

Bracken fern (*Pteridium aquilinum*) is a higher plant, with a high content of mutagenic, clastogenic and immunosuppressive chemicals, related to naturally-occurring cancer in animals (G. Borzacchiello, 2003). The main carcinogen of bracken fern – ptaquiloside – acts synergistically with the E5 protein of BPV-2, contributing for the mesenchymal carcinogenesis in the urinary bladder (Martano et al., 2013). Thus, although BPV-2 has an important role in these cattle neoplasias, the presence of environmental cofactors is crucial for the development of bladder tumors (G. Borzacchiello, 2003).

BPV-2 infection and Chronic Enzootic Haematuria (CEH) syndrome – a urinary bladder neoplasia associated with bracken fern ingestion – are frequently linked (Pamukcu, Prince, & Bryan, 1976). BPV DNA has been detected in the urinary bladder urothelium of CEH affected animals and of healthy cattle, suggesting a latent or sub-clinical infection (S Roperto et al., 2010). In bovines, CEH leads to the development of tumors in the bladder wall, causing severe haematuria (Carvalho, Pinto, & Peleteiro, 2006).

Some authors refer to the possibility of productive infection in peripheral blood mononuclear cells (PBMCs) in cattle (Sante Roperto et al., 2011, 2012). The same authors provided evidence of BPV-2 presence and productive infection in the placenta of cows with bladder cancer, which might imply the potential to compromise the placental function, leading to reproductive disorders (Sante Roperto et al., 2012). However, due to the fact of BPV-1 and BPV-2 replication and virion production only occur in the epithelial cells, it is difficult to unquestionably accept the above enunciated possibilities.

1.6 Vaccination

The fact that the virus life cycle is restricted to the epithelium, sheltered from the immune system, is the primary reason for the poor immune response to BPV. However, when the papilloma is damaged, causing bleeding tumors, BPV antigens come into contact with the host immune cells that consequently develop high antibodies titers against the virus. Nevertheless, there is a prolonged immune response when viral proteins are used to immunize the host. Vaccines can be prophylactic or therapeutic. Prophylactic vaccines induce protection from the infection or/and disease, and Therapeutic vaccines induce regression of early tumors (M S Campo, 2003).

Initially, an attempt was made to vaccinate calves by the inoculation of viral particles. This type of vaccination has presented results in the prevention of infection, and in the production of neutralizing antibodies. The conferred immunity was type-specific, i.e., calves were protected only from the BPV type used in the vaccine (Giuseppe Borzacchiello & Roperto, 2008; M S Campo, 1997).

The L1 capsid protein was used in a vaccine against BPV-2, triggering the production of neutralizing antibodies and the prevention of infection. Along with L1, the L2 protein was also used in a vaccine against BPV-2 in cattle, resulting in a regression of warts. However, by vaccination with L2, the induced anti-L2 antibodies were not neutralizing (Giuseppe Borzacchiello & Roperto, 2008; M S Campo, 1997), binding to the virus particles but not neutralizing the virus infectivity.

When the L1 protein is expressed in eukaryotic cells, it has the ability to self-assemble into a virus-like particle (VLP), without genetic material inside. This structure is highly similar to the virus virion, and is capable to induce an immune response when used as a vaccine (Giuseppe Borzacchiello & Roperto, 2008). The PV VLPs can induce a strong immune response from B cells, even with VLPs low doses and without adjuvants (Chackerian, Lowy, & Schiller, 2001). For HPV, two VLP vaccines are available; one HPV vaccine version is constituted by VLPs of HPV-16, HPV-18, HPV-6 and HPV-11, four mucosal HPVs. The second HPV vaccine version is constituted by VLPs of HPV-16 and HPV-18 (M Saveria Campo & Roden, 2010).

When using BPV-2 VLP vaccines, it is possible to induce neutralizing antibodies against BPV-1 in cattle, as well as BPV-1 VLP vaccines may also induce neutralizing antibodies against BPV-2. In this way, a BPV-1 or BPV-2 VLPs monovalent vaccine would be able to induce protection against the homologous and heterologous virus. These results are evidences of the closely serotype relatedness between BPV-1 and BPV-2 (Shafti-Keramat et al., 2009).

In contrast with studies that mentioned the inability of PV early genes to induce a protective response from the host, another study has demonstrated that the BPV-4 E7 protein induced immunization by rejection of established tumors in calves, although it did not prevented infection. It was also seen a strong cellular immune response by the host when the E7 vaccine was used – in spontaneous regressing BPV-4 papillomas, large infiltrates of activated lymphocytes have been detected, mostly $CD4^+$ T-cells (Brun & Riethmuller, 2007; M S Campo, 1997). The vaccinated animals also shown an increase of anti-E7 antibodies (Giuseppe Borzacchiello & Roperto, 2008).

BPV has been frequently used as a model to study HPV, and its investigation has proved to be essential to clarify the oncogenic potential of the virus, the interaction between PVs and environmental/biological factors, and to develop new vaccines. The mechanisms that are beyond PVs infection and carcinogenesis are also enlightened by the study of BPV (Giuseppe Borzacchiello & Roperto, 2008). The expression and characterization of oncoproteins, and structural proteins, have the potential to offer important biotechnological products, from antibodies to potential vaccines (Mazzuchelli-de-Souza et al., 2013).

Chapter 2

MATERIAL AND METHODS

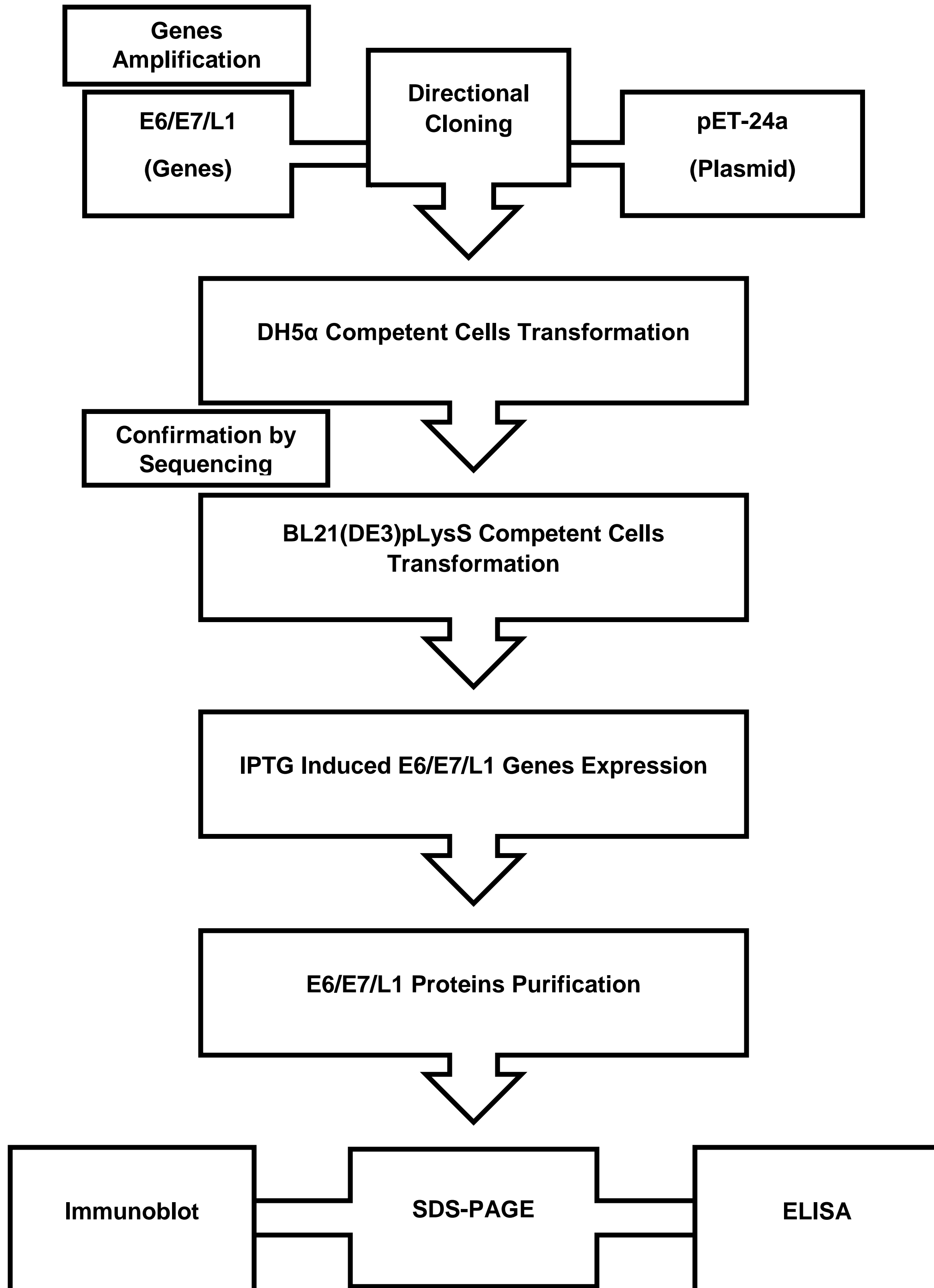

Figure 4 - Representing scheme of the methodology for cloning and expression of E6, E7 and L1 genes of BPV-2.

2.1 E6, E7 and L1 Genes Amplification

For the E6 gene amplification, an E6 previously cloned sequence on the pGEM®-T vector (Promega®) was used as template. The E7 and L1 sequences were amplified from DNA samples of BPV-2 positive papillomas, collected from a papillomatosis affected bovines. The BPV-2 positive samples were kindly provided by João Cota, a PhD student of the Faculty of Veterinary Medicine.

Forward and reverse primers were designed to include in the 5' end different restriction sites, allowing a directional cloning into the plasmid expression vector pET-24a (Novagen®). Each gene sequence was previously analyzed for restriction mapping, using the CLC Main Workbench Program (CLCBio, Qiagen®), to guarantee that the restriction sites chosen were absent within the gene sequence.

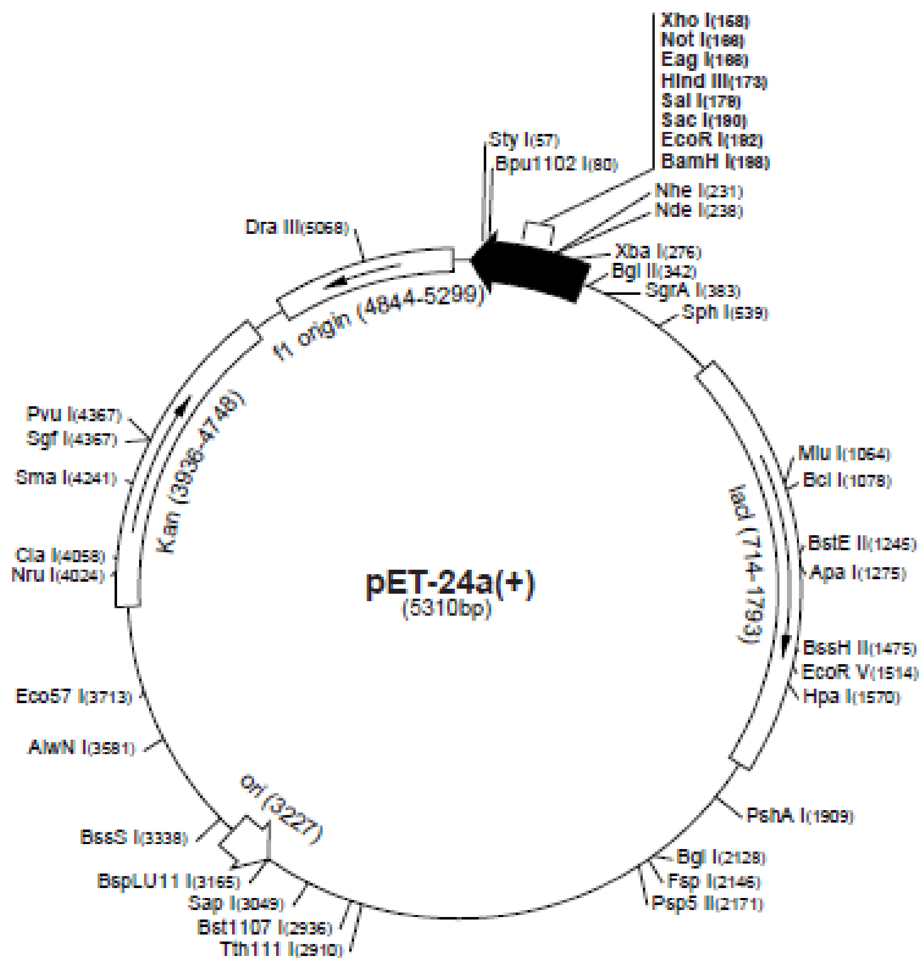

Figure 5 - Map of the plasmid vector pET-24a (Novagen®).

For E6 and E7 genes the *NheI/XhoI* pair were chosen and for the L1 the pair *EcoRI/XhoI*. Previous to each restriction site a two/three nucleotide tail was added, according to the NEB-Guide (Neb, 2011), to

fulfill the restriction enzyme requirement. In the forward primer, the restriction site was immediately followed by the initiation codon ATG of the gene. In the reverse primer, the restriction site was followed by the specific gene terminal sequence, excluding the stop codon (Table 1).

Table 1 - Primers used for E6, E7 and L1 genes amplification. The underlined sequences represent the 5' flanking nucleotides (NEB-Guide (Neb, 2011)); the bold/underlined sequences represent the restriction endonucleases recognition sites, followed by the specific gene sequences of BPV-2.

	Forward Primer	**Reverse Primer**
E6	***NheI*** CTA**GCTAGC**ATGGACCTGCAAAGTTTTTCC	***XhoI*** CCG**CTCGAG**TGGGTAGTTGGACCTTGAACC
E7	***NheI*** CTA**GCTAGC**ATGGTTCAAGGTCCAACTACC	***XhoI*** CCG**CTCGAG**TCGTTTGCCATGACGCTC
L1	***EcoRI*** CG**GAATTC**ATGGCGTTGTGGCAACAAGGCCAG	***XhoI*** CCG**CTCGAG**AGCTTTGATTTTTTTTCTTTTTGCAGGC

E6 (414bp) and E7 (384bp) genes were amplified using 1x Master Mix 2.5x (5 Prime®), and 0.8µM of each primer in a 25µl reaction with approximately 50ng of recombinant plasmid DNA for E6, and 500ng of genomic DNA for E7. The cycling conditions included 10 minutes at 95ºC for initial denaturation, followed by 35 cycles at 95ºC for 30 seconds, 58ºC for 30 seconds, 68ºC for 1 minute, and a final elongation step at 68ºC for 10 minutes.

The L1 gene (1494bp) amplification was performed in a 50 µl reaction, using 1x Master Mix 2.5x (5 Prime®), 0.8µM of each primer and 500ng of genomic DNA. The cycling conditions included an initial denaturation step of 10 minutes at 95ºC, followed by 40 cycles at 95ºC for 15 seconds, 60ºC for 30 seconds, 68ºC for 45 seconds and a final elongation step at 68ºC for 10 minutes. For the negative controls the same PCR mix without template DNA was used.

All amplifications were performed on a Doppio thermal cycler (VWR®).

The presence of the specific amplicons was confirmed by agarose gel electrophoresis of 10µL of the amplification reaction with 1x Loading buffer (50% Glycerol, 20% 0.5M EDTA, 0.05% Bromophenol Blue, 0.05% Xylene cyanol and H_2O), in a 1,5% agarose gel stained with 0,005% GelRed Nucleic Acid Gel Stain (Biotium®), in 1x TAE (40mM Tris acetate, 1mM EDTA). NZYDNA ladder V (NZYTech®) for E7

amplicon, and NZYDNA ladder III (NZYTech®) for E6 and L1 amplicons, were used as molecular-weight size markers. The electrophoresis gels were run at constant voltage (90V) until full resolution of the amplicons, and visualized in a transilluminator (Pharmacia Biotech ImageMaster® VDS, Pharmacia Biotech®) with UV light.

The specific amplicons (E6 – 414bp, E7 – 384bp and L1 – 1494bp) were purified using the kit DNA Clean & Concentrator™-5 (ZYMO RESEARCH®), according to the manufacters instructions, and eluted in 11µL of DNA Elution Buffer. The purified DNA was quantified in a NanoDrop 2000c (THERMO SCIENTIFIC®) and stored at -20ºC until use.

2.2 Hydrolysis and Ligation

For directional cloning into the expression plasmid – pET-24a (Novagen®), both plasmid and amplicons were double hydrolyzed with the two restriction enzyme pairs.

E6 and E7 amplicons were hydrolyzed with the restriction enzymes *XhoI* and *NheI* whereas the L1 amplicon was hydrolyzed with the restriction enzymes *EcoRI* and *XhoI*. The reactions were performed with fast digest restriction enzymes (NZYTech®), according to the manufacturer's instructions, in a final volume of 20µL. For the hydrolysis was used 200ng of E6, E7 and L1 amplicons, and 1000ng of pET-24a.

The reaction mixture was incubated at 37ºC for 1 hour for E6, E7 and L1 amplicons hydrolysis, and for 5 minutes for pET-24a plasmid hydrolysis, followed by an incubation at 65ºC for 5 minutes in a QBD Block heater (Grant®), for enzyme inactivation. The hydrolyzed product was purified using DNA Clean & Concentrator™-5 kit (ZYMO RESEARCH®), eluted in 11µL of DNA elution buffer and quantified in a NanoDrop 2000c (THERMO SCIENTIFIC®).

The ligation was performed in 20µL reaction, using a Speedy Ligase (NZYTech®), following the manufacturer's instructions: 25% Speedy ligation buffer 4x (NZYTech®) and 5% Speedy Ligase (NZYTech®). To calculate the correct vector/insert molar ratios the following equation (pGEM®-T and pGEM®-T Easy Vector Systems Guide (Promega, 2010)) was used to determine the necessary ng of the insert, considering 50ng of plasmid vector:

$$\frac{ng\ of\ vector\ x\ kb\ size\ of\ insert}{kb\ size\ of\ vector}\ x\ insert{:}vector\ molar\ ratio = ng\ of\ insert$$

(Equation 1)

The reaction was incubated for 1 hour at room temperature and stored at 4ºC, until use.

2.3 Preparation of Competent Cells

Competent DH5α (*F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ–*) and BL21(DE3)pLysS (*F- ompT gal dcm lon hsdSB(rB- mB-) λ(DE3) pLysS(cmR)*) cells were prepared from a colony of each bacterial strain. Each colony was inoculated in 5mL of LB broth, without antibiotics, and incubated at 37ºC over-night, at 250RPM/minute.

From the 5mL culture, 1mL was used to inoculate 100mL of LB broth and incubated at 37ºC and 25RPM/minute until OD_{600}=0.45 – 0.55. The culture was aliquoted in 50mL Falcons, incubated on ice for 10 minutes, and centrifuged at 4200Xg for 10 minutes, at 4ºC. The pellets were washed twice in 10mL of 50mM $CaCl_2$, and centrifuged at 3500Xg, for 10 minutes, at 4ºC and kept in ice for 30 minutes before centrifuged at 2800Xg, for 5 minutes, at 4ºC. The cells were suspended in 2mL of 100mM $CaCl_2$ and 15% Glycerol, aliquoted in pre-refrigerated microtubes and stored at -80ºC until use.

2.4 Transformation of DH5α and BL21(DE3)pLysS Competent Cells

After ligation of E6, E7 and L1 with pET-24a, DH5α competent cells were transformed. Briefly, 5µL of the ligation product was added to 50µL of competent cells previously thawed on ice.

The mixture was incubated on ice for 20 minutes, followed by a heat-shock at 50ºC for 90 seconds in a QBD Block heater (Grant®) and kept on ice for 2 minutes.

The bacterial cells were incubated in a 50mL Falcon tube with 950µL of SOC medium (NZYTech®) at 37ºC for 1 hour in a Gallenkamp Orbital Incubator at 200RPM/minute. After centrifugation at 1000Xg for 10 minutes, the pellet was resupended in 200µL of SOC medium (NZYTech®) and plated in two LB agar (NZYTech®) plates with 50µg/mL kanamycin, each with 100µL of bacterial cells, and incubated overnight at 37ºC.

The resulting bacterial colonies were screened by conventional PCR, under the same conditions described previously (section 2.1) to confirm transformation with each recombinant plasmid. Positive colonies were picked and seeded in 10mL of LB broth (NZYTech®) with 50µg/mL kanamycin and incubated overnight at 37ºC in a Gallenkamp Orbital Incubator at 200RPM/minute; 1mL aliquots of the liquid culture were diluted in LB/glycerol (1:1) and stored at -80ºC and 5mL were used for the recombinant plasmid DNA extraction using ZR Plasmid Miniprep™ kit (ZYMO RESEARCH®), according to the manufacters instructions. The plasmid DNA was sequenced by Sanger sequencing (Stabvida, Portugal) to confirm the insertion and specificity of the recombinant sequence.

The recombinant plasmid DNA was later used to transform BL21(DE3)pLysS competent cells, following the above described protocols used for DH5α™ transformation, screening and stocking. For BL21(DE3)pLysS transformation, the plates were supplemented with 50µg/mL kanamycin and 50µg/mL chloramphenicol.

2.5 IPTG Induced Expression E6, E7 and L1 Genes

Protein expression of BPV-2 E6, E7 and L1 genes was carried out, according to the Novagen pET System Manual, and The QIAExpressionist Handbook.(Novagen, 2003; Qiagen, 2001) Briefly, a 10ml starter culture was grown in LB broth (NZYTech®) with 50µg/mL kanamycin and 50µg/mL chloramphenicol overnight at 37ºC in a Gallenkamp Orbital Incubator at 200 RPM/minute; 3mL were collected and seeded in 50mL of LB broth (NZYTech®) with the same antibiotics and grown at 37ºC in a Gallenkamp Orbital Incubator at 200RPM/minute until the cell density reached OD_{600}=0.5 – 1.

To induce the E6, E7 and L1 expression, IPTG (NZYTech®) was added to the culture, to a final concentration of 1mM. Prior to induction, a 2mL aliquot was collected. The culture was grown for 4 hours at 37ºC in a Gallenkamp Orbital Incubator at 200RPM/minute, and 2mL aliquots were collected hourly. Each aliquot was centrifuged at 1000Xg for 5 minutes, and the pellet and supernatant were separately stored at -20ºC.

After a 4 hour incubation period, the culture was centrifuged at 4000Xg for 20 minutes, and the pellet was stored at -20ºC.

2.6 Western Blot Analysis

At this stage, a Western Blot analysis was performed to evaluate the protein expression profile of the induction cultures. Western Blot analysis include the electrophoretic separation of proteins by SDS-PAGE (**S**odium **D**odecyl **S**ulphate - **P**oly**a**crilamide **G**el **E**lecrophoresis), electrical transfer of the gel proteins to a PVDF (***p**oly**v**inylidene **d**i**f**luoride*) membrane and detection of the expressed protein using specific antibodies. SDS-PAGE and protein transfer was done in the Mini-PROTEAN® Tetra Handcast system (Bio Rad®) and Mini Trans-Blot® Cell system (Bio Rad®), respectively.

2.6.1 SDS-PAGE Analysis and Protein Quantification

The SDS-PAGE technique allows the electrophoretic separation of proteins by its size, and not by its charge and conformation due to the presence of SDS (***s**odium **d**odecyl **s**ulfate*), β-Mercaptoethanol and a heat shock. Protein samples are resuspended in a loading buffer, β-Mercaptoethanol and SDS. Prior to electrophoresis the sample is heated to 100-96ºC for 10-5 min, for protein denaturation and binding of SDS, providing a global negative charge uniformizing the charging differences between proteins. With a negative uniform charge, sample proteins will migrate towards the positive electrode, according to their molecular weight.

To analyze the levels of the recombinant protein in BL21(DE3)pLysS induced cells, two fraction were collected:

a) Total Cell Protein Fraction;

b) Insoluble Cytoplasmic Fraction (Inclusion Bodies).

In order to extract the expressed protein from inclusion bodies, the induced cells from each collection point were hydrolyzed overnight in 10mM Tris-HCL (pH=8), 1M NaCl, 50mM Urea, 1M β-Mercaptoethanol and 20mM Imidazole, in a Movil-Rod (Selecta Group®). The digested cells were centrifuged for 20 minutes at 13.5RPM in a Hermle Z 383 K centrifuge (HERMLE Labortechnik®), and stored at -20ºC.

The lysed solution was later purified in a His Spin Trap column (GE Healthcare®), since an histidine-tag in the amino end of the expressed proteins. The column was washed with 20mM Tris-HCl, 8M Urea, 500mM NaCl, 5mM Imidazole and 1mM β-Mercaptoethanol, and was eluted twice with 20mM Tris-HCl, 8M Urea, 500mM NaCl, 500mM Imidazole and 1mM β-Mercaptoethanol. Each protein elution aliquots were quantified by the Bradford method, using a calibration curve (Annex B). The purified protein was stored at -20ºC, until SDS-PAGE.

To evaluate the protein expression along time, 5µL of each protein sample was resuspended in 15µL of 4x SDS-PAGE buffer (25% 0,5M Tris-HCL, 20% Glycerol, 40% SDS (10%), 10% β-Mercaptoethanol and 5% Bromophenol blue), and denatured at 100ºC for 10 minutes in a QBD Block heater (Grant®). Samples were run in a 15% polyacrylamide gel: Acrylamide (40%) (NZYTech®), 25% 1.5M Tris-HCl (pH=8.8), 1% SDS (10%), dH_2O, 0.57% APS (10%) and 0.03% TEMED at 200V for 1hour, in 1x Running Buffer (5x) (25mM Tris base, 192mM Glycine, 0.1% SDS and dH_2O). The NZYColour Protein Marker II (NZYTech®) was used as protein molecular weight ladder.

The SDS-PAGE gel was stained with 25mL of BlueSafe (NZYTech®), for protein visualization.

2.6.2 Transfer to PVDF Membranes

For western blot, a parallel gel was run and transferred to 0.2µm PVDF membrane (invitrogen®) (Figure 6), in 1x Transfer buffer (25mM Tris base, 192mM Glycine, 10% Methanol and dH_2O) at 40mA overnight.

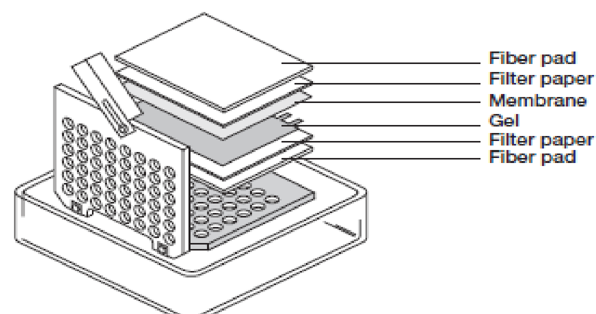

Figure 6 - Schematic representation of the system used for the protein transfer from a polyacrylamide gel to a PVDF membrane. (BIORAD Mini Trans-Blot®, Instruction Manual).

2.6.3 Immunoblotting

This technique relies on the capacity of specific antibodies to recognize and bind to specific protein antigens. For detection of the His-tag recombinant proteins, an Anti-His Mouse IgG was used as primary antibody, and an Anti-Mouse IgG Peroxidase conjugated, as secondary antibody.

To avoid unspecific binding, the PVDF membrane was previously incubated in Blocking buffer (10% (w/v) non-fat dry milk, 1x PBS (SIGMA-ALDRICH®) and 0.05% Tween™-20), for 4-5 hours at room temperature with gentle shaking.

The primary antibody was diluted in the Blocking/Incubation buffer (10% (w/v) non-fat dry milk, 1x PBS (SIGMA-ALDRICH®) and 0.05% Tween™-20), and incubated with the membrane for 1 hour at room temperature with gentle shaking. Posteriorly, the membrane was washed twice with 20mL of Washing Buffer (PBS and 0.05% Tween™-20), for 5 minutes at room temperature with gentle shaking. The secondary antibody was diluted in the Blocking/Incubation buffer and incubated for 1 hour at room temperature with gentle shaking. For signal detection a 4-Chloro-1-naphthol solution (83mM 4-Chloro-1-naphthol, 0.06% H_2O_2 and 96% TBS) was added, until color development.

Table 2 - Primary and secondary antibodies used for the Immunoblotting of E7 and L1 proteins. The working dilutions of the primary and secondary antibodies are indicated.

	Primary Antibody	Secondary Antibody
E7	Anti-E7 Rabbit IgG (1:1000)	Anti-Rabbit IgG/PO (1:3000)
	Anti-His Mouse IgG (GE Healthcare®) (1:3000)	Anti-Mouse IgG/PO (SIGMA-ALDRICH®) (1:50000)
L1	BPV-2 positive bovine serum (1:10)	Anti-Bovine IgG/PO (working dilution 1:1000)
	Anti-His Mouse IgG (GE Healthcare®) (1:3000)	Anti-Mouse IgG/PO (SIGMA-ALDRICH®) (1:50000)

2.7 ELISA

ELISA (**E**nzyme-**L**inked **I**munno**s**orbent **A**ssay) is an immunological assay that allows the detection of linked antigen/antibody complexes, through an enzymatic reaction, similarly to the immunoblot assay.

Costar™ 96-Well EIA/RIA Plates (Fisher Scientific®) wells were coated with 200ng of protein extract/100µL 1x PBS per well, and incubated at 4ºC overnight; washed five times with 100µL 1x PBS; blocked with 100µL of PBS-BSA 3% per well and incubated at 37ºC for 1 hour. The plate was washed five times with 1x PBS per well and 100µL of primary antibody were added to each well in duplicates; the plate was incubated at 37ºC for 1 hour. After a final washing step, the conjugated secondary antibody was added and the plate was incubated at 37ºC for 1 hour.

Once the final incubation was performed, the plate was washed 5 times with 1x PBS, and 100µL of substrate solution (ABTS – *2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)*) plus 1.7% H_2O_2 (30%)) were added to each well, until color development. The optical density was recorded at 405nm at different time points in a SpectraMAX 340PC microplate reader (MOLECULAR DEVICES®).

Table 3 - Primary and secondary Antibodies used in the ELISA technique for E7 and L1 proteins. The working dilution for each antibody is indicated.

	Primary Antibody	**Secondary Antibody**
E7	Anti-E7 Rabbit IgG (1:500; 1:1000; 1:5000; 1:10000; 1:50000; 1:60000; 1:80000; 1:100000)	Anti-Rabbit IgG/PO (1:3000)
L1	BPV-2 positive cow serum (1:10)	Anti-Bovine IgG/PO (1:1000)

Chapter 3

RESULTS

3.1 Evaluation of the E6 and E7 Genes Expression

3.1.1 PCR amplification of E6 and E7 gene fragments

The E6 gene sequence (414bp) was amplified from a previously cloned recombinant plasmid (pGEM®-T vector) sample. For the E7 gene (384bp) amplification, several DNA samples of BPV-2 positive animals were used.

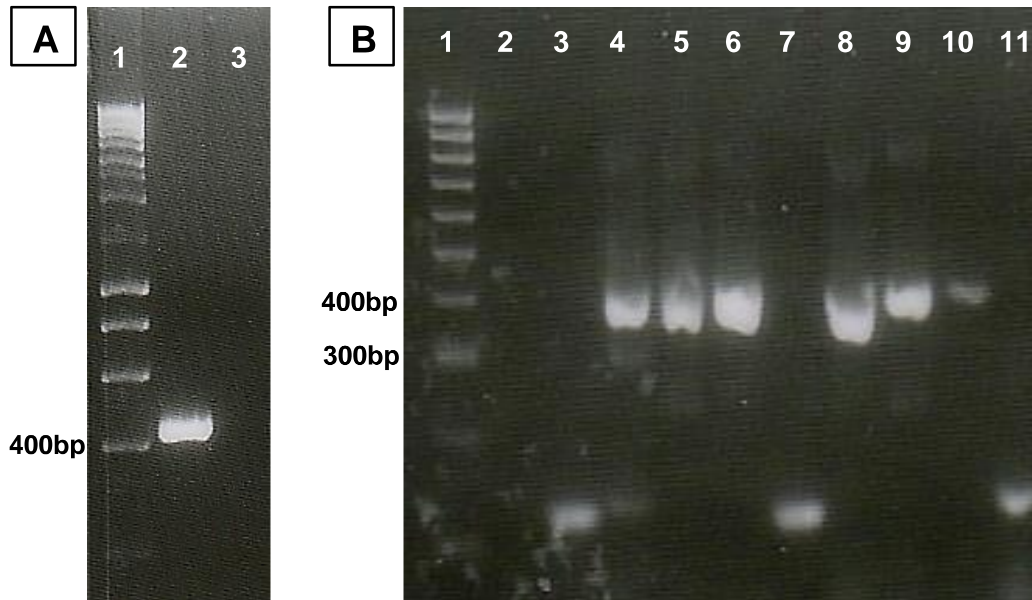

Figure 7 – E6 (A) and E7 (B) amplicons in a 1.5% Agarose Gel Electrophoresis. A: Lane 1: NZYDNA Ladder III (NZYTech®), Lane 2: E6 PCR amplification product, Lane 3: Negative control. B: Lane 1: NZYDNA Ladder V (NZYTech®), Lane 2: Negative Control, Lanes 3-11: E7 PCR amplification products.

The E6 gene was successfully amplified with the designed primers, yielding the expected band with the correct molecular-weight (414bp) (Figure 7 - **A**). For the E7 gene amplification a 384bp amplicon was expected (Figure 7 – **B**), and obtained from BPV-2 positive samples. For the E7 pET-24a cloning only one of the amplicons was used.

3.1.2 Screening of transformed DH5α cells with the recombinant plasmids (pET-24a/E6 and pET-24a/E7)

Although pET-24a includes a kanamycin resistance selective marker, it does not include the coding sequence of β-galactosidase within the Multiple Cloning Site, disallowing blue and white colony screening after cell transformation in the presence of IPTG and X-Gal. To confirm the presence of the

recombinant plasmid (pET-24a/E6, pET-24a/E7), after transformation in the selective medium with kanamycin, a colony PCR was performed with the same primers used for E6 and E7 PCR amplification, to discriminate between plasmid and recombinant plasmid transformed cells.

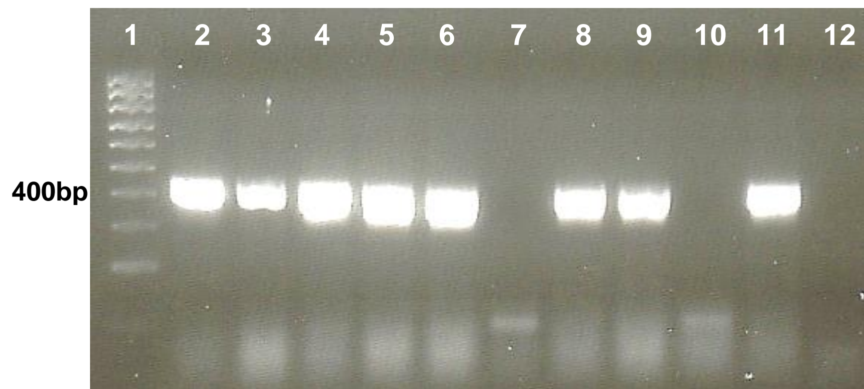

Figure 8 – Colony screening of recombinant plasmids pET-24a/E6. Lane 1: NZYDNA Ladder V (NZYTech®), Lanes 2-11: Screening PCR products, Lane 12: Negative Control.

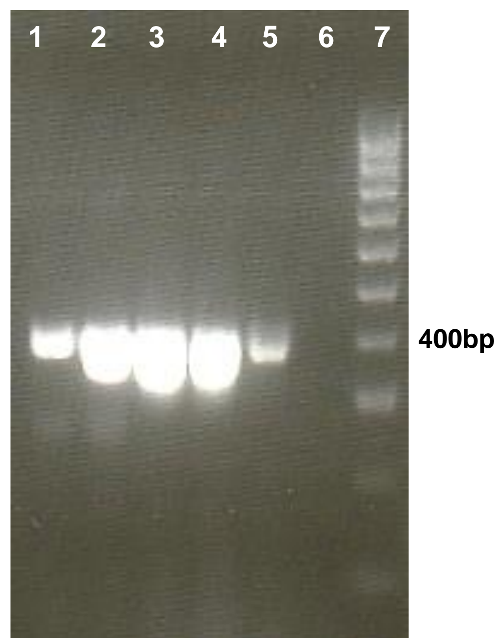

Figure 9 – Colony screening of recombinant plasmids pET-24a/ E7. Lanes 1-5: Screening PCR products, Lane 6: Negative Control, Lane 7: NZYDNA Ladder V (NZYTech®).

Several colonies tested positive for each gene (Figure 8: pET-24a/E6; Figure 9: pET-24a/E7), confirming the successful transformation with each recombinant plasmid.

After recombinant plasmid DNA extraction for each gene, from one chosen colony, the insert specificity and orientation was confirmed by Sanger sequencing. The sequencing results showed that E7 was correctly cloned inside the pET-24a plasmid. However, for E6, only the sequence with the forward sequencing primer was obtained, showing an adenine insertion immediately downstream the forward

primer sequence (Figure 10). Even with a negative E6 sequencing result, both E6 and E7 extracted plasmids were used to transform BL21(DE3)pLysS competent cells.

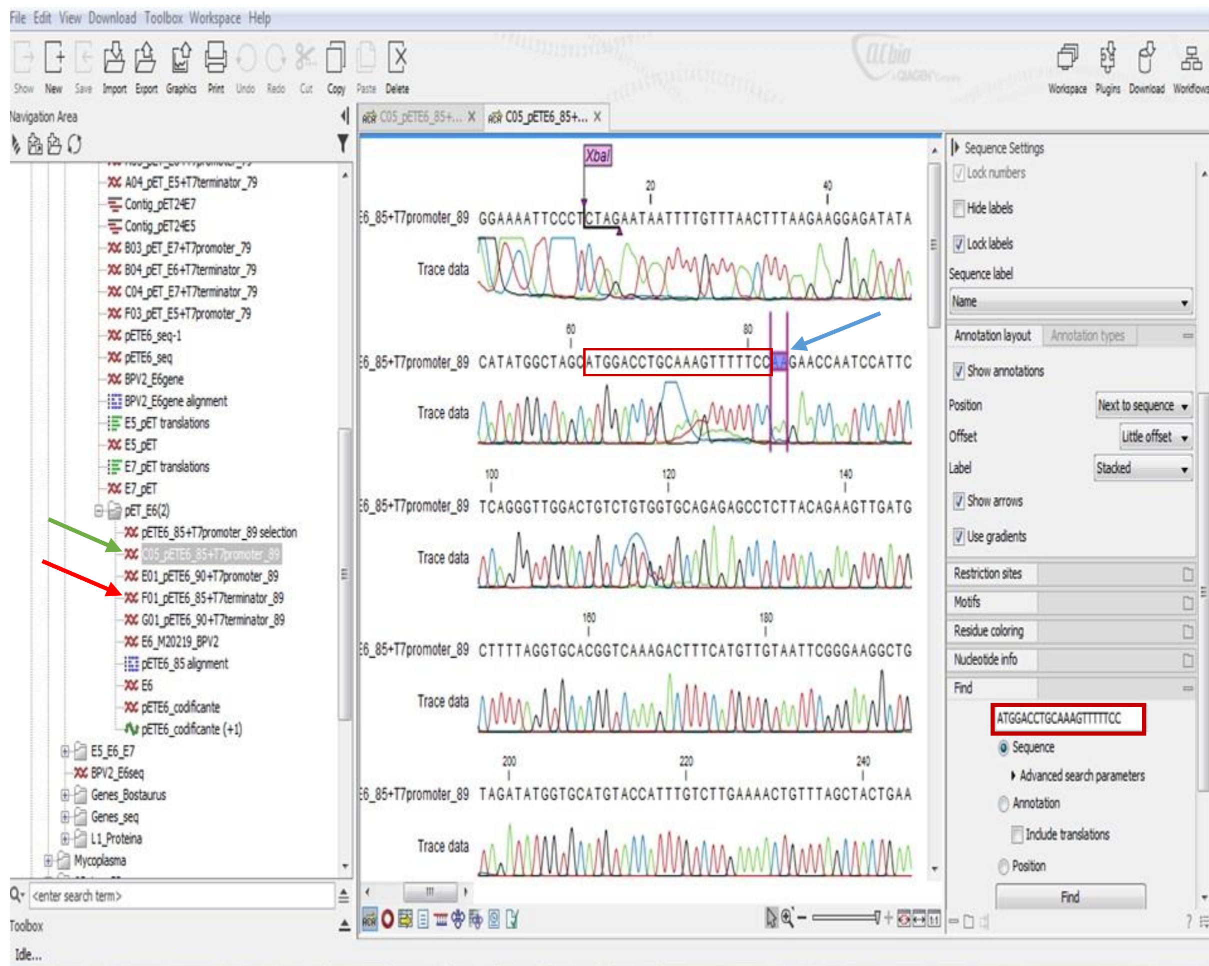

Figure 10 – E6 sequencing chromatogram obtained with the forward sequencing primer. The forward sequencing primer sequence is highlighted within the red rectangle. Blue arrow: Local of nucleotide (adenine) insertion; Green arrow: Sequence obtained with the forward sequencing primer; Red arrow: Sequence obtained with the reverse sequencing primer.

3.1.3 SDS-PAGE analysis of the E6 and E7 expressed proteins

After IPTG induced expression, each time point (t0; t1; t2; t3; t4) of the solubilized samples was analyzed in a 15% polyacrylamide gel.

The E6 corresponding protein (15.8kDa) was not detected (Figure 11 – **A**). The E7 (13.6kDa) protein band was detected from t1 to t4 with increasing intensity (Figure 11 – **B**).

Due to the high protein diversity of the protein extract, the E7 samples were purified in His Spin Trap columns (GE Healthcare®) and were run in a SDS-PAGE (Figure 12).

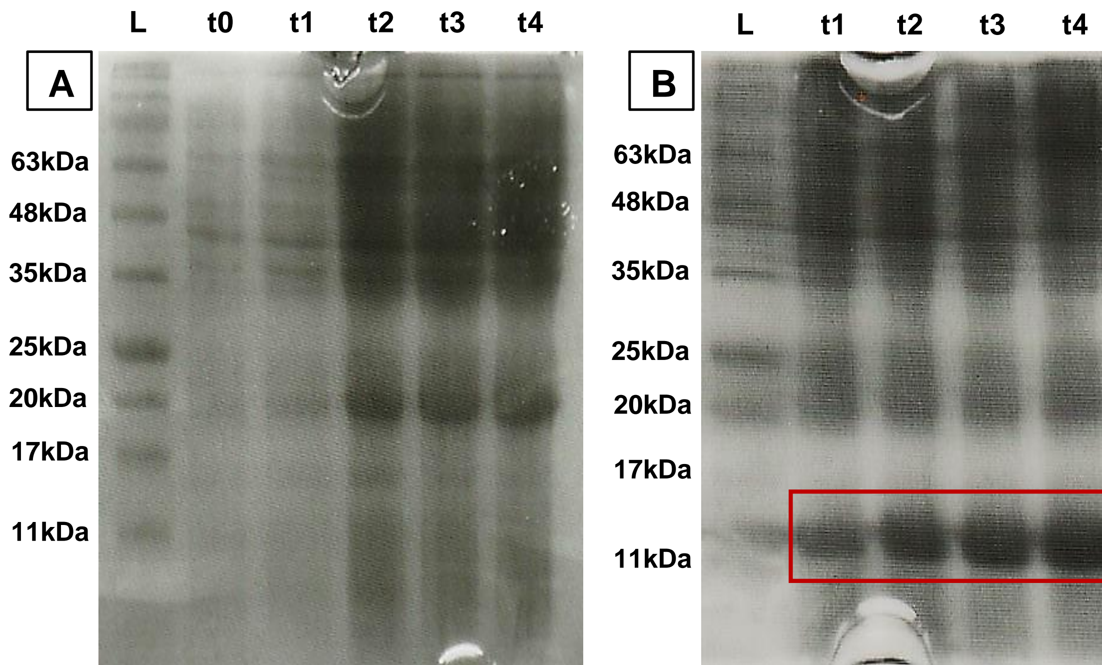

Figure 11 – Polypeptide profile of the induced E6 (15.8kDa) (A) and E7 (13.6kDa) (B) protein samples in transformed BL21(DE3)pLysS cells by 15% Polyacrylamide Gel, before purification. A: L: NZYColour Protein Marker II (NZYTech®), t0: Sample of 0 hours induction, t1: Samples of 1 hour induction, t2: Sample of 2 hours induction, t3: Sample of 3 hours induction, t4: Sample of 4 hours induction. B: L: NZYColour Protein Marker II (NZYTech®), t1: Sample of 1 hour induction, t2: Sample of 2 hours induction, t3: Sample of 3 hours induction, t4: Sample of 4 hours induction. The putative E7 protein is highlighted within the red rectangle.

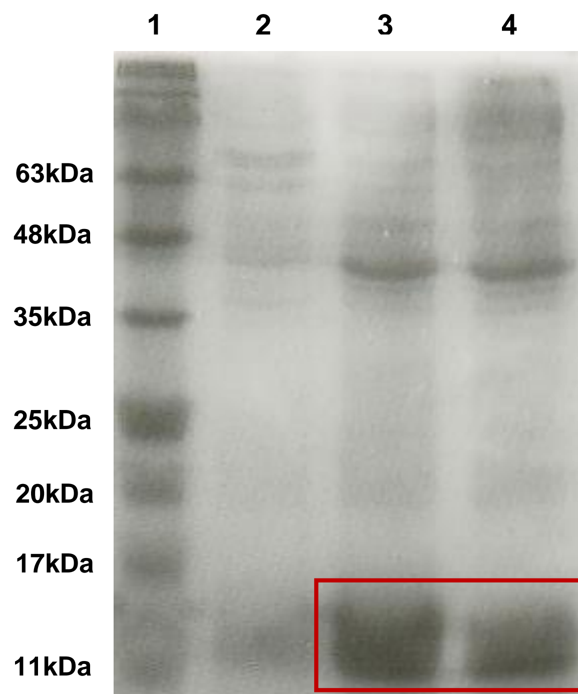

Figure 12 - Polypeptide profile of the purified E7 sample by 15% Polyacrylamide Gel. Lane 1: NZYColour Protein Marker II (NZYTech®), Lane 2: Sample from after the first washing step, Lane 3: Samples from the first elution step, Lane 4: Sample from the second elution step. The E7 protein is highlighted within the red rectangle.

A protein band of E7 expected molecular weight was detected in the first and second elution steps (Figure 12: lanes 3 and 4).

3.1.4 Immunoblot of E7 protein

To confirm the specificity of the 13.6kDa protein band, an immunological detection was performed, using a rabbit Anti-E7 antibody (DeMasi et al., 2005), kindly provided by Professor Peter Howley, Harvard Medical School, and an Anti-Rabbit IgG/PO as secondary antibody, with positive staining of the corresponding E7 protein band (Figure 13).

Furthermore, in order to detect the protein by its histidine tail, an Anti-His Mouse IgG was used as primary antibody. However, no visible bands were seen, and therefore no image is presented.

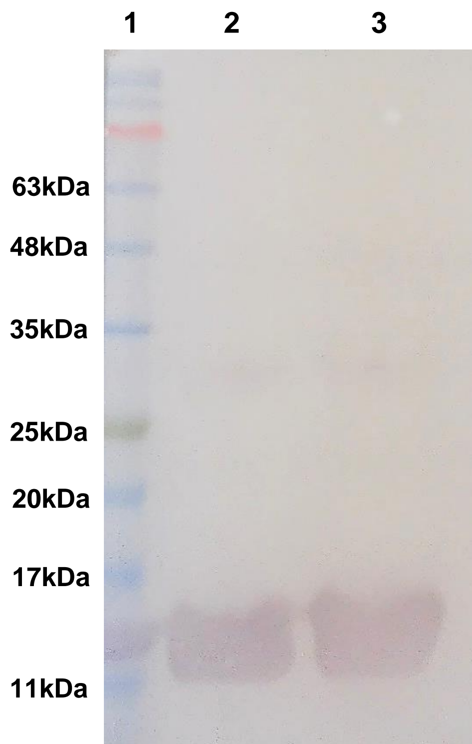

Figure 13 – Immunoblotting of E7 protein (13.6kDa) in PVDF membrane (invitrogen®) using Anti-E7 Rabbit IgG as primary antibody and Anti-Rabbit IgG as secondary antibody. Lane 1: NZYColour Protein Marker II (NZYTech®), Lane 2-3: E7 protein.

3.1.5 ELISAs for E7 protein detection

The specificity of the recombinant E7 protein was further assessed by ELISA using the rabbit anti-E7 IgG (Figure 14; Table 4).

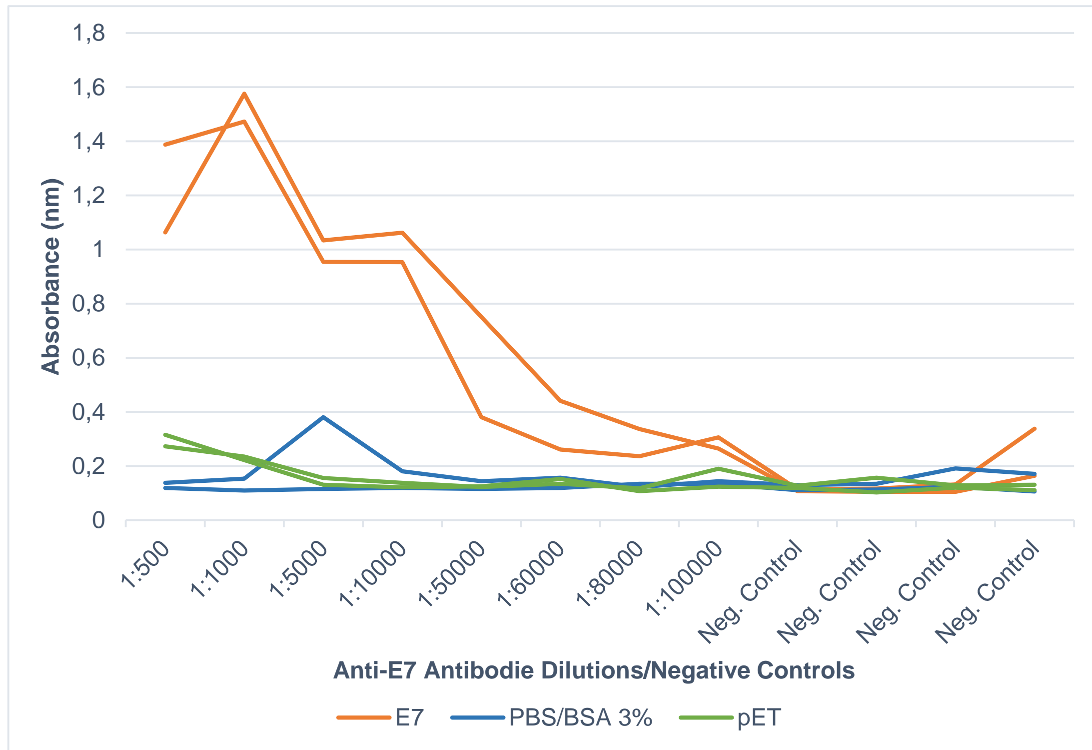

Figure 14 – Diagram of the ELISA results when using an Anti-E7 rabbit IgG as primary antibody (1:500; 1:1000; 1:5000; 1:10000; 1:50000; 1:60000; 1:80000 and 1:100000) and an Anti-Rabbit IgG/PO as secondary antibody. The ELISA was performed in wells coated with the expressed E7 protein, non-recombinant pET and Blocking Buffer (PBS/BSA 3%). For the negative controls (Background Controls), coated wells were incubated only with the secondary antibody. The presented results were obtained 10 minutes after incubation with the substrate solution.

In the wells coated with the E7 antigen, an expressive absorbance signal was detected from the 1:500 up to 1:100000 Anti-E7 rabbit IgG dilution. However, when using a 1:500 dilution of Anti-E7 IgG, the absorbance signal was lower than the one registered at 1:1000 dilution, which can be explained by a reaction inhibition due to the high titer of primary antibody at 1:500, i.e., prozone effect. Furthermore, at 1:1000 dilution of Anti-E7 IgG, the absorbance signal reached its maximum (Absorbance≈1.50).

In the wells incubated with the secondary antibody (Negative Controls – Background Controls), no absorbance signal was recorded, confirming the non-binding of the secondary antibody with the tested antigen. Additionally, the signals registered in E7-coated wells, at all Anti-E7 rabbit IgG dilutions, were superior to the signals registered in the control wells (non-recombinant pET and blocking buffer wells).

Table 4 – Absorbance values of the duplicates obtained in the indirect ELISA. Anti-E7 rabbit IgG was used as primary antibody and anti-Rabbit IgG/PO as secondary antibody. Plates were coated with the expressed E7 protein, non-recombinant pET and Blocking Buffer (PBS/BSA 3%). For background evaluation, coated wells were incubated directly with the secondary antibody. The presented results were obtained 10 minutes after incubation with the substrate solution.

	Anti-E7 rabbit IgG dilutions								Negative Controls (No primary antibody)			
	1:500	1:1000	1:5000	1:10000	1:50000	1:60000	1:80000	1:100000				
E7	1.38	1.47	0.95	0.95	0.37	0.26	0.23	0.30	0.11	0.11	0.13	0.33
	1.06	1.57	1.03	1.06	0.75	0.44	0.33	0.26	0.10	0.10	0.10	0.16
Blocking Buffer	0.13	0.15	0.37	0.18	0.14	0.15	0.12	0.14	0.12	0.13	0.19	0.17
	0.11	0.10	0.11	0.11	0.11	0.11	0.13	0.13	0.11	0.11	0.12	0.10
Non-recombinant pET	0.27	0.23	0.15	0.13	0.12	0.13	0.11	0.18	1.18	0.15	0.12	0.13
	0.31	0.22	0.11	0.11	0.12	0.15	0.10	0.12	0.99	0.10	0.11	0.10

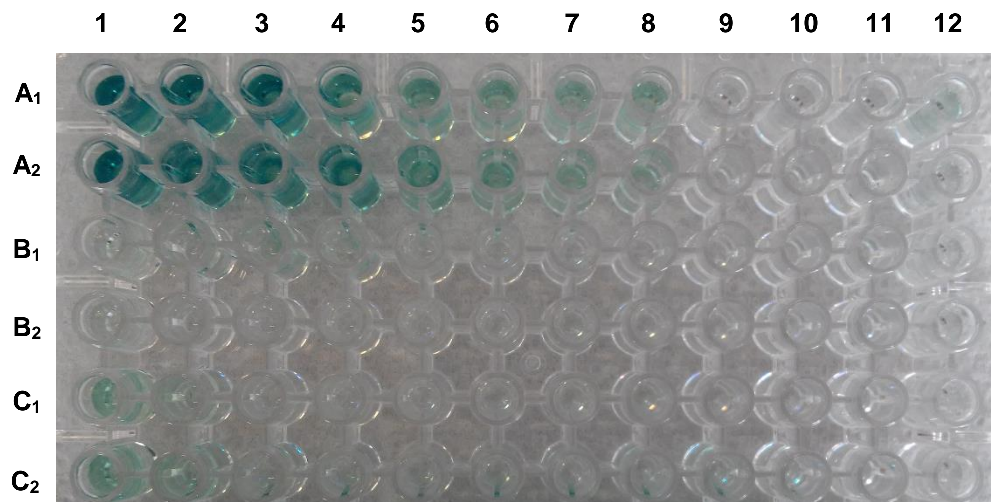

Figure 15 – ELISA plate, when using an Anti-E7 rabbit IgG as primary antibody and an Anti-Rabbit IgG/PO. The ELISA was performed in wells coated with the expressed E7 protein, non-recombinant pET and Blocking Buffer (PBS/BSA 3%). For the negative controls, coated wells were incubated only with the secondary antibody. The presented results were obtained 10 minutes after incubation with the substrate solution. A_1: E7 replica 1, A_2: E7 replica 2, B_1: blocking buffer replica 1, B_2: blocking buffer replica 2, C_1: non-recombinant pET replica 1, C_2: non-recombinant pET replica 2; Anti-E7 rabbit IgG dilutions: Lane 1: 1:500, Lane 2: 1:1000, Lane 3: 1:5000, Lane 4: 1:10000, Lane 5: 1:50000, Lane 6: 1:60000, Lane 7: 1:80000, Lane 8: 1:100000; Lane 9-12: Negative controls.

The specificity of the anti-E7 IgG was also tested, using an HIV Integrase (Figure 16).

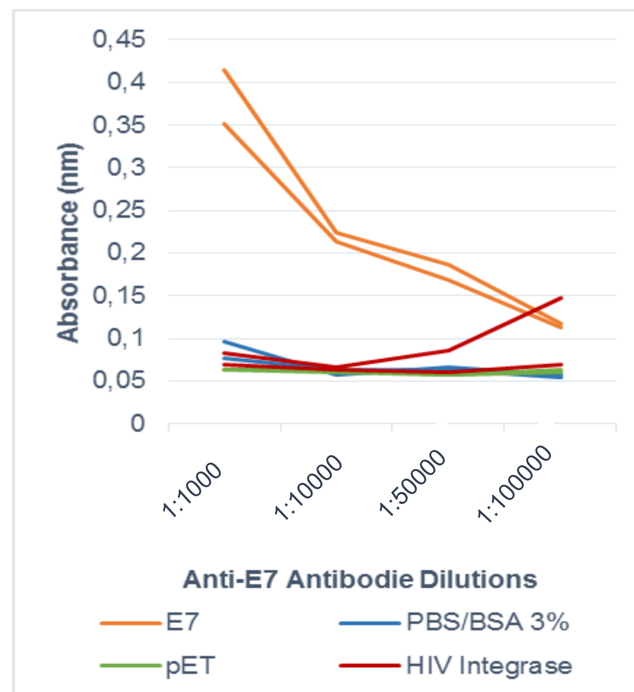

Figure 16 – Diagram of the ELISA results when using an Anti-E7 rabbit IgG as primary antibody and Anti-Rabbit IgG/PO as secondary antibody. The ELISA technique was performed in wells coated with the expressed E7 protein, non-recombinant pET, Blocking Buffer (PBS/BSA 3%), and an HIV integrase exogenous protein. The presented results were obtained 10 minutes after incubation with the substrate solution.

Only the E7 protein coated wells recorded a significant absorbance signal, confirming the Anti-E7 IgG specificity to the E7 protein. The detection of absorbance signal in one of the HIV integrase replica can be explained by faulty washing steps.

3.2 Evaluation of L1 Gene Expression

3.2.1 PCR amplification of L1 Gene Fragment

For the L1 gene (1494bp) amplification, a DNA sample of a PBV-2 positive animal was used, yielding the expected band with the correct molecular weight (Figure 17).

Due to the presence of mispriming in the amplification reaction, the L1 gene fragment was purified directly from the agarose gel.

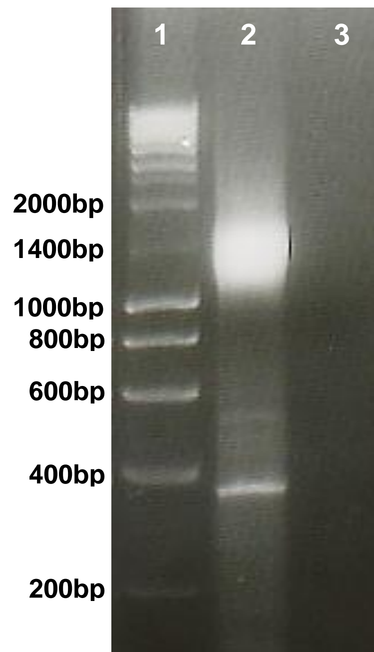

Figure 17 – L1 amplicon in a 1.5% Agarose Gel Electrophoresis. Lane 1: NZYDNA Ladder III (NZYTech®), Lane 2: L1 PCR amplification product, Lane 3: Negative control.

3.2.2 Screening of transformed DH5α cells with the recombinant plasmid pET-24a/L1

The screening of recombinant plasmid transformed DH5α cells was performed with the procedure used for the E6 and E7 (Section 3.1.2). A colony PCR was performed with the same primers used for L1 PCR amplification, allowing the discrimination between plasmid and recombinant plasmid transformed cells.

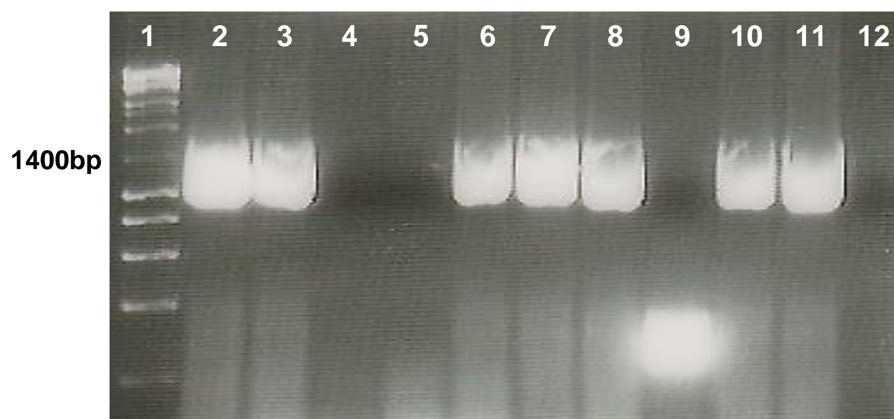

Figure 18 - Colony screening of recombinant plasmids pET-24a/L1 in DH5α cells. Lane 1: NZYDNA Ladder III (NZYTech®), Lanes 2-11: Screening PCR products, Lane 12: Negative Control.

Several of the colonies tested by PCR for L1 confirmed the transformation with recombinant plasmid pET-24a/L1 (Figure 18).

An additional experimental test was performed to confirm that the bands were in fact correspondent to the cloned L1 gene. Plasmid DNA was extracted and hydrolyzed with the same restriction enzymes used for L1 cloning, resulting in the excision of the L1 gene fragment, with the correct molecular weight, from the vector plasmid (Figure 19).

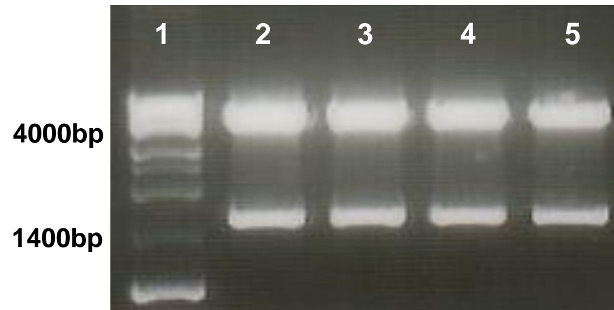

Figure 19 – Hydrolysis of recombinant plasmids pET-24a/L1. Lane 1: NZYDNA Ladder III (NZYTech®), Lanes 2-5: Hydrolyzed DNA products.

The extracted recombinant plasmid DNA, from one chosen colony, was Sanger sequenced. The L1 sequencing results confirmed the insert specificity and correct orientation. The extracted plasmid DNA was later used to transform BL21(DE3)pLysS competent cells.

3.2.3 Screening of transformed BL21(DE3)pLysS cells with the recombinant plasmid pET-24a/L1

The screening of BL21(DE3)pLysS cells with the recombinant plasmid (pET-24a/L1) was also performed, using the same procedure described for DH5α screening (Section 3.2.2).

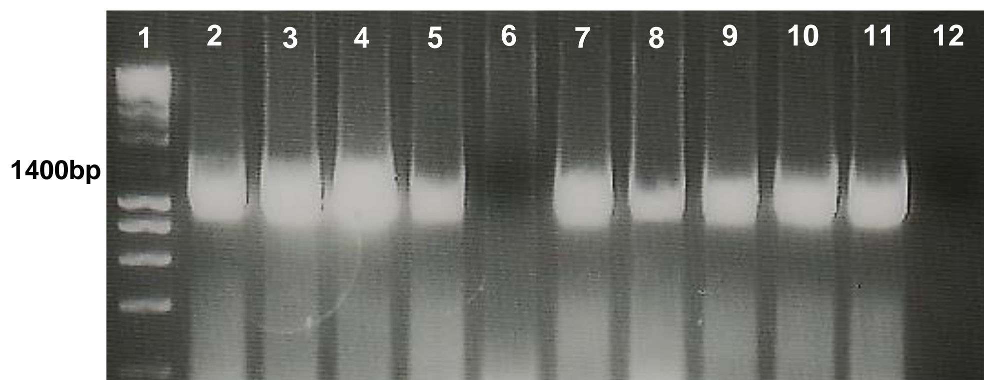

Figure 20 - Screening of recombinant plasmids pET-24a/L1 in BL21(DE3)pLysS cells by colony amplification. Lane 1: NZYDNA Ladder III (NZYTech®), Lanes 2-11: Screening PCR products, Lane 12: Negative Control.

The successful transformation of BL21(DE3)pLysS with the recombinant plasmid (pET-24a/L1) was confirmed through the analysis of several colonies (Figure 20).

3.2.4 SDS-PAGE analysis of L1 gene expression

Similarly to E7 expression analysis, after IPTG induced expression of L1 (55.5kDa), each time point (t0; t1; t2; t3; t4) of the solubilized samples was analyzed in a 1.5% polyacrylamide gel.

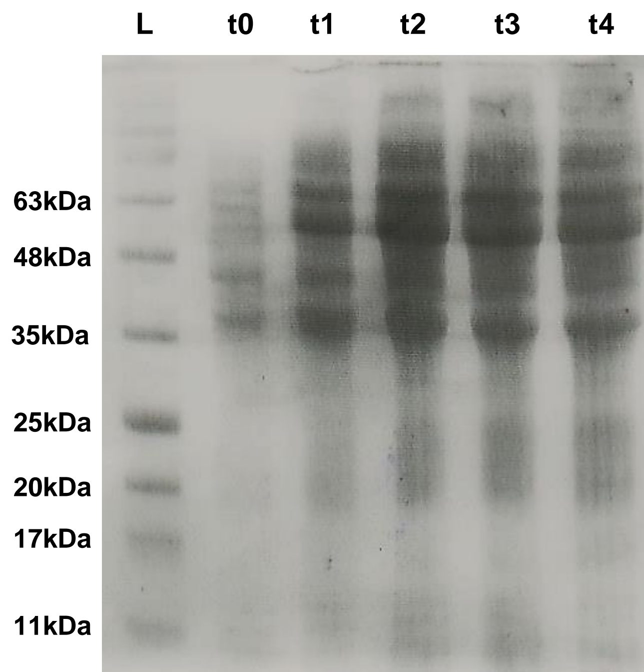

Figure 21 - Polypeptide profile of the induced L1 (55.5kDa) protein samples in transformed BL21(DE3)pLysS cells by 15% Polyacrylamide Gel, before purification. L: NZYColour Protein Marker II (NZYTech®), t0: Sample of 0 hours induction, t1: Sample of 1 hour induction, t2: Sample of 2 hours induction, t3: Sample of 3 hours induction, t4: Sample of 4 hours induction.

A band with 55.5kDa was expected in the polyacrylamide gel (Figure 21), however due to the poor resolution of the gel and the number of proteins within this image, it was difficult to confirm the presence of L1 protein band. The samples were purified in a His Spin Trap column (GE Healthcare®) and run in a SDS-PAGE (Figure 22).

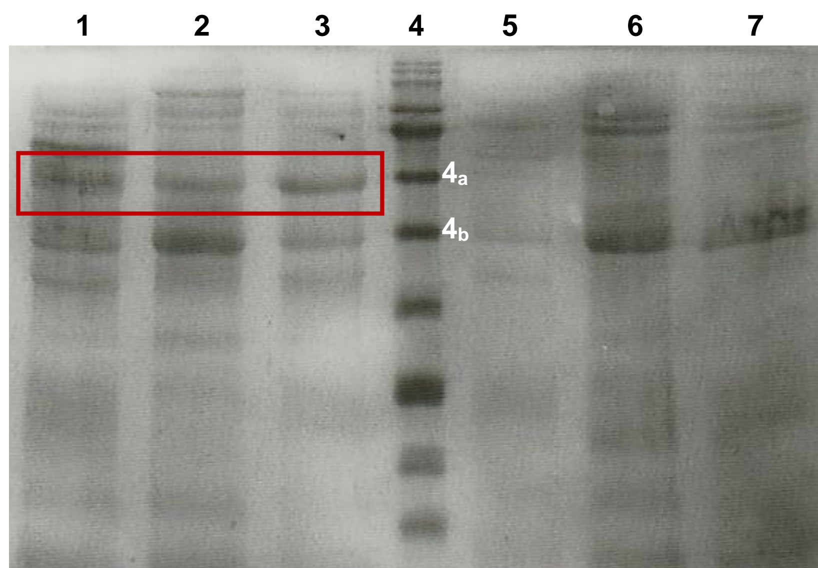

Figure 22 – Polypeptide profile of the purified L1 (Left) and non-recombinant pET (Right) samples by 15% SDS-PAGE. Left: Lane 1: Sample from the purification washing step, Lane 2: Sample from the first elution step, Lane 3: Sample from the second elution step. Lane 4: NZYColour Protein Marker II (NZYTech®). Right: Lane 5: Sample from the purification washing step, Lane 6: Sample from the first elution step, Lane 7: Sample from the second elution step. 4_a: 63kDa, 4_b: 48kDa. The putative L1 protein is highlighted within the red rectangle.

After purification, a band of the putative L1 protein (55.5kDa) was visualized in lanes 1, 2 and 3 (Figure 22 – Left). The same bands were not present in the non-recombinant pET samples, reinforcing the hypothesis of a correct L1 protein expression (Figure 22 – Right).

However, observation of a band with the compatible size (highlighted in Figure 22) was not enough to assume that L1 was actually expressed, and an immunoblotting was performed in order to confirm the protein expression.

In immunoblotting, using BPV-2 positive cow serum as primary antibody, no visible bands were detected. Also, an Anti-His Mouse IgG was used as primary antibody in an Immunoblot experience. Once again, no results were obtained.

3.2.5 ELISA for L1 protein

An ELISA methodology was followed in order to clarify the uncertainty regarding the protein expression. Due to the lack of a specific antibody against the L1 protein, a BPV-2 positive cow serum was used to analyze the putative L1 expressed protein. Knowing that the presence of anti-*E. coli* antibodies in the serum could mask the ELISA results, BPV-2 positive cow serum was pre-incubated with non-recombinant pET samples, in order to reduce its anti-*E.coli* antibodies concentration (Figure 23; Table 5).

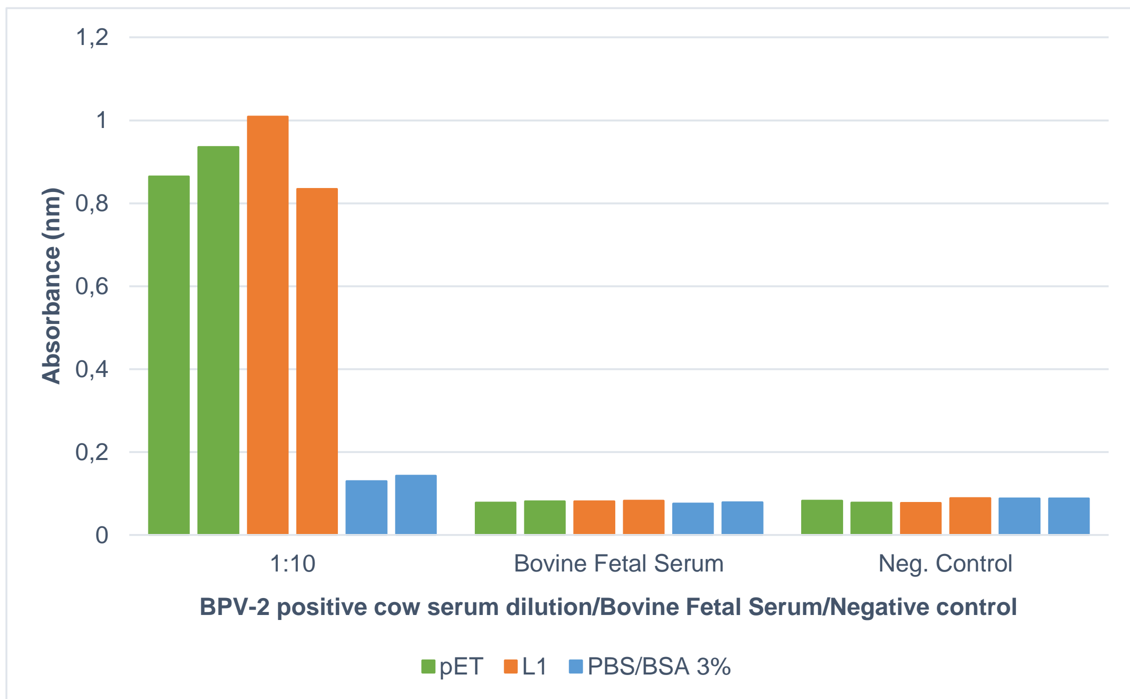

Figure 23 – Comparison of the ELISA results using BPV-2 positive cow serum and Bovine Fetal Serum as primary antibodies, and Anti-Bovine IgG as secondary antibody. The ELISA technique was performed in wells coated with the expressed L1 protein, non-recombinant pET and Blocking Buffer (PBS/BSA 3%). For the negative control (Background Controls), the BPV-2 positive cow serum wasn't used, being only used the Anti-Bovine IgG secondary antibody. The presented results were obtained 20 minutes after incubation with the substrate solution.

As expected, when the serum was used in wells coated with the Blocking Buffer (PBS/BSA 3%), the absorbance values were extremely low. The Bovine Fetal Serum was also used as primary antibody and, due to the lack of antibodies against BPV-2 in this serum, the registered absorbance values were also extremely low.

When using a BPV-2 positive cow serum as primary antibody, a similar absorbance value was detected between L1 and the non-recombinant pET.

The similar absorbance signal recorded both in L1 and non-recombinant pET did not allow to confirm the presence of the protein in the purified extracts. However, the strategy used to reduce the anti-*E. coli* antibodies titer in the serum was inefficient, and further improvement of the methodology could lead to more convincing results.

Table 5 - Absorbance values registered in ELISA performed with BPV-2 positive cow serum and Bovine Fetal Serum as primary antibodies, and Anti-Bovine IgG as secondary antibody. The ELISA technique was performed in wells coated with the expressed L1 protein, non-recombinant pET and Blocking Buffer (PBS/BSA 3%). For the negative control, only the Anti-Bovine IgG secondary antibody was used. The presented results were obtained 20 minutes after incubation with the substrate solution.

	BPV-2 positive cow serum (1:10)	Blocking Buffer	Negative Controls (No primary antibody)
Non-recombinant pET	0.86	0.07	0.08
	0.93	0.08	0.07
L1	1.00	0.08	0.07
	0.83	0.08	0.08
Blocking Buffer	0.12	0.07	0.08
	0.14	0.07	0.08

Chapter 4

DISCUSSION

The experimental work developed in this thesis was centered in the amplification, cloning and expression three genes of Bovine papillomavirus type 2 – E6, E7 and L1. The recombinant clones were later used to express the corresponding proteins. The expressed proteins were analyzed by immunoblotting and ELISA, in order to confirm their presence and specificity. The L1 protein was also used to investigate the presence of anti-L1 antibodies in BPV-2 positive cow serum.

For this investigation, the same methodology was used for all the three genes expression cloning. However, the results for each gene were divergent, since only for E7, the presence and specificity of a specific protein was demonstrated.

4.1 E6 Results Discussion

E6 is one of the oncogenes present in the viral genome. The E6 protein is associated with cell transformation and immortalization (Corteggio et al., 2013). This gene was cloned into the expression plasmid – pET-24a, and sequenced, using the same primers of the PCR amplification. The sequencing results suggested that the gene was not cloned in the correct frame, due to a nucleotide insertion immediately after the forward primer sequence, which would shift the open reading frame of the gene, resulting in a different protein. A proofreading taq polymerase was used, and due to its proofreading activity, hardly it would be responsible for a nucleotide insertion.

However, this insertion could be due to a misreading in the sequencing reaction, and no sequencing was obtained with the plasmid reverse sequencing primer, which may also indicate that cloning was not successfully achieved. For this reason the recombinant plasmid was used for BL21(DE3)pLysS cells transformation and its expression induced and evaluated.

After expression, the cell extracts were solubilized and analyzed in a SDS-PAGE. No protein with the expected MW (15.8kDa) was detected, confirming the unsuccessful cloning.

In order to overcome this problem, it would be necessary to re-clone the gene and repeat the process from the beginning.

4.2 E7 Results Discussion

E7 is an oncogene located in the early region of BPV-2 genome. The E7 protein has a transforming activity, and is highly important in apoptotic signaling (G Borzacchiello et al., 2009; Corteggio et al., 2011; DeMasi et al., 2007, 2005).

The E7 gene was amplified, hydrolyzed and cloned inside pET-24a. The sequencing results have shown that the gene was properly inserted inside the plasmid vector, and the protein expression of the recombinant plasmid was induced. In the SDS-PAGE analysis, it was possible to visualize a protein of

the expected molecular weight – 13.6kDa in the recombinant plasmid protein extract. The corresponding protein band was absent in the protein extract of transformed pET-24a *E. coli*.

Protein purification in His Spin Trap columns, resulted in a decrease of unspecific background proteins levels. This purification method relies on the presence of a histidine tail in the expressed E7 protein. A decrease in the protein background can be interpreted as an indirect form of confirming the expression and presence of the protein.

The use of an anti-E7 antibody was crucial to confirm that E7 was present in the expression samples. An immunoblotting experiment was done, using the anti-E7 Rabbit IgG as primary antibody, and the result was positive.

An Anti-His Mouse IgG was also used in the immunoblotting experiment, but no visible band was detected. It is possible that a low specificity of the Anti-His IgG as the result of antibody degradation, a low sensibility of the detection system due to non-optimized conditions in the experiment procedure, may have accounted for this negative result. Further protocol adjustments may be necessary to obtain better results.

An ELISA technique was also performed, using the anti-E7 Rabbit IgG as primary antibody. When using the anti-E7 antibody in ELISA wells containing E.coli proteins, i.e., in wells coated with protein extract of non-recombinant pET-24a, the absorbance signal was considerably lower than the signal recorded in E7 protein samples. The anti-E7 Rabbit IgG/E7 antigen complex formation is essential for signal emission, which confirms the presence of E7 in the protein extracts.

Although the E7 expression was performed in a prokaryotic system (*E. coli* BL21(DE2)pLysS cells), which presents different translational mechanisms from an eukaryotic system (*e.g.*, absence of post-translational modifications), the protein conserved its antigenic epitopes. The anti-E7 Rabbit IgG was produced against an E7 protein, expressed in the same prokaryotic system – *E. coli* BL21(DE2)pLysS cells (DeMasi et al., 2005). For this reason, the ligation between the E7 protein and the anti-E7 Rabbit IgG was expected.

The obtained E7 protein may be used for specific and monoclonal antibodies production, which could be further used to investigate and enlighten the role and mechanisms of E7 in infected cells. An investigation conducted by João Cota, a PhD student of the Faculty of Veterinary Medicine, was centered in eukaryotic cells transfection with several BPV-2 genes, including E7; hence, anti-E7 antibodies would also be useful to investigate the mechanisms behind E7 in the transfected cells.

These antibodies could also be used as diagnostic tools in order to assess the replicative stage of the virus infection in different cells.

4.3 L1 Results Discussion

The L1 protein is the major structural protein that constitutes the PV virion capsid and, together with L2, is essential in the virus infectivity (Lunardi et al., 2013). For this reason, this protein has been extensively investigated, as a vaccine candidate, but also to clarify the virus requirements for effective infection. These two proteins – L1 and L2 – are expressed in the most differentiated layers of host's epithelium, being indispensable in the virion structure assembly. L1 has also the ability to self-assemble in a virus-like particle (VLP), deprived from genetic material; and its use as vaccines candidates have proven highly important (Buck et al., 2013). HPV is considered as the main causal agent in cervical cancer (Franco & Harper, 2005). Two types of vaccines are used to prevent cervical cancer, and both are based on HPV VLPs, which are important for the reduction of HPV related diseases (Wang & Roden, 2013b). When comparing to live and attenuated vaccines, VLP-based vaccines are considerably safer, which makes them ideal investigation targets nowadays (Wang & Roden, 2013b).

The L1 gene was amplified from a BPV-2 positive sample, hydrolyzed and cloned into pET-24a. The sequencing results showed that the gene was cloned in the correct frame within the plasmid vector and its expression was IPTG induced. SDS-PAGE analysis showed the presence of a protein band which size was within the expectable molecular mass (55.5kDa), but these results were not conclusive. Due to the presence of a histidine tail in the recombinant protein, the protein extract was purified, using HisSpinTrap columns, with a substantial decrease in the background proteins, which was suggestive of the specificity of the putative 55.5kDa protein band.

An ELISA was developed, using pET-24a *E.coli* protein extract and pET-24a/L1 protein extract coated wells. Serum of BPV-2 positive cow was used, assuming the presence of specific anti L1-IgG but also anti-*E.coli* IgG. To reduce the expected background due to the anti-*E. coli* IgG, the serum was pre-incubated in pET-24a *E.coli* protein extract coated wells, and transferred for the pET-24a/L1 protein extract coated wells. However, the background clearance was not achieved, and we could not reach any conclusion regarding: i) the presence of specific anti-L1 IgG in the serum of a BPV-2 positive animal and consequently, ii) the identification of the putative L1 protein band. Further on, a more efficient method for protein purification should be used, in order to isolate the expressed protein from the background proteins. High performance liquid chromatography (HPLC) and Immunoaffinity chromatography are techniques that could be used, in order to efficiently purify the protein.

In parallel with the ELISA and to further clarify this issue, an immunoblotting was performed using serum of the BPV-2 positive cow, against the purified L1 putative protein band, but no visible bands were detected.

The absence of conclusive results regarding the L1 protein expression may be due to lack of expression of a viral protein, coded by a eukaryotic virus, in a prokaryotic system. The inexistence of post-translational modifications in prokaryotes could be a factor that inhibited the expression of the viral protein in the prokaryotic system (*E. coli* BL21(DE3)pLysS cells). Although the sequencing data pointed to a successful cloning, the protein expression in a prokaryotic system may have proven unsuccessful,

despite of the presence of protein bands with the expected molecular mass in the electrophoretic analysis. The availability of a specific antibody (anti-L1 IgG) would help solving this doubt. The virus infection do not cause a strong immune response in the host (M S Campo, 2003), and therefore the anti-L1 antibodies present in the BPV-2 positive cow serum may have a low titer, and for this reason it would be difficult to detect them. Also, the presence of anti-*E. coli* antibodies, possible at much higher concentration, may have blurred the presence of anti-L1 IgG.

However, the use of a BPV-2-positive cow serum, with papillomatosis, would be an expectable source of anti-L1 IgG, due to the inflammatory reaction in the affected skin, allowing the presentation of viral antigens to the immune system. Another possibility for the absence of L1 identification would be changes in the protein conformation due to the denaturing conditions used in the SDS-PAGE, invalidating its recognition by specific anti-L1 antibodies.

The recombinant DNA clones could be expressed in a eukaryotic system (yeast or insect cells) in order to produce L1 VLPs. The VLPs could be posteriorly used to produce BPV-2 and BPV-1 vaccines due to the fact of being two closely related serotypes (Chackerian et al., 2001; Shafti-Keramat et al., 2009). The obtainment of a BPV-2 VLP vaccine would be an important step towards the treatment and prevention of BPV-2 related tumors and infection.

The procedure that was followed and the results obtained in this thesis did not lead to a dead-end, and it is possible to glimpse a path from here. In the future, the knowledge that was obtained in these experiences may be useful to facilitate the successful expression of the E6 protein. Furthermore, with access to an anti-L1 antibody it would be possible to clarify the doubts that have emerged in its expression.

The gene sequences have been successfully amplified and, further on, its nucleotide sequences may be analyzed by phylogenetic studies.

The E5 protein is another oncoprotein of PV that is extremely important in the oncogenic mechanisms of the virus (Vande Pol & Klingelhutz, 2013), which makes it an ideal candidate to study and investigate.

The expression of recombinant oncoproteins allows the development of valuable diagnostic tools (Mazzuchelli-de-Souza et al., 2013), to investigate the dynamic of the viral infection in the infected animals. The DNA tumor virus oncoproteins have been frequently used to identify cellular proteins and pathways that are important to cell transformation, such as proliferation, apoptosis and signal transduction (DeMasi et al., 2005). Thus, expression and posterior purification of the viral oncoproteins, as the objective of this experimental work was, are crucial to identify and investigate the mechanisms and pathways in cell transformation.

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Annexes

Annex A – Nucleotide sequences of E6, E7 and L1 genes:

The genes sequences was retrieved and can be accessed by the following NCBI accession number: *M20219.1*.

E6: (5’-ATGGACCTGCAAAGTTTTTCCAGAGGCAATCCTTTCTCAGGATTGGCCTGT GTTTGGTGC AGGGAGCCTCTCACAGAAGTTGATGCTTTTAGGTGCATGATAAAAGACTTTCATGTTGTATACCGA GATGGTGTGAAATTTGGTGCATGTACCACTTGTCTTGAGAACTGCTTAGATAAAGAAAGAAGACT GTGGAAAGGTGTGCCAGTAACAGGTGAGGAAGCTCAATTATTGCATGGCAAATCCCTTGATAGGC TTTGCATAAGATGCTGCTACTGTGGGGGAAAACTAACCAAAAACGAGAAGCAGCGGCATGTGCTT TATAATGAGCCTTTTTGCAAAACGAGATCTAACATAATAAGAGGACGCTGCTACGACTGCTGCAG ACATGGTTCAAGGTCCAACTACCCATAG-3’);

E7: (5’-ATGGTTCAAGGTCCAACTACCCATAGAAACTTGGATGATTCACCTGCAGGACCGTTGCTG ATTTTAAGTCCATGTGCAGGCACACCTACCAGGGTTCCTGCAGCACCTGATGCACCCGATTTCAG ACTTCCGTGCCATTTCGGCCGTCCTACTAGGAAGCGAGGTCCCTCTACGCCTCCGCTTTCCTCTC CCGGAAAAGTGTGTGCAACAGGGCCACGTCGAGTGTACTCTGTGACTGTCTGCTGCGGACACTG CGGAAAGGACCTTACATTTGCTGTCAAGACTGGCTCTACGACCTTGCTGGGCTTCGAACACCTAT TAAACTCAGATTTGGACCTGTTGTGTCCCCGTTGCGAATCTCGCGAGCGTCATGGCAAACGATAA -3’);

L1: (5’-ATGGCGTTGTGGCAACAAGGCCAAAAGCTGTATCTCCCTCCAACCCCTGTAAGCAAGGT GCTATGCAGTGAAACCTATGTGCAAAGAAAAAGCATATTCTATCATGCAGAAACGGAACGCCTGT TAACTGTAGGACATCCATACTACCAAGTCACTGTGGGGGACAAAACTGTTCCCAAAGTGTCTGCT AATCAATTTAGAGTTTTTAAAATACAGCTCCCCGATCCCAATCAGTTTGCATTGCCTGATAGGACT GTGCACAATCCAAGCAAGGAGCGCCTGGTTTGGGCTGTAATAGGGGTTCAAGTATCTCGTGGCC AACCACTAGGAGGCACAGTTACTGGGCACCCCACTTTTAATGCTCTGCTTGATGCAGAAAATGTT AATAGAAAAGTTACTGCACAAACAACAGATGACAGGAAGCAAACAGGATTAGATGCTAAGCAACA ACAGATTCTGTTGCTGGGCTGTACCCCTGCAGAAGGGGAATACTGGACCACAGCCCGTCCATGT GTTACTGATAGACTAGAAAATGGTGCGTGTCCTCCTTTAGAATTAAAGAACAAACACATAGAAGAT GGAGACATGATGGAAATAGGGTTTGGTGCTGCTGACTTTAAAACACTAAATGCCAGTAAATCAGA TCTACCTCTTGACATTCAAAATGAAATATGCCTGTATCCAGACTACCTCAAAATGGCTGAAGATGC TGCTGGAAACAGTATGTTCTTCTTTGCAAGAAAAGAACAAGTGTATGTAAGGCATATATGGACTCG GGGGGGCTCTGAAAAAGAAGCACCCAGTAAAGACTTCTACCTCAAAAATGGTAGAGGTGAAGAA ACTCTAAAAATACCTAGTGTGCACTTTGGCAGTCCCAGTGGATCCTTGGTGTCCACTGATAATCAA ATATTTAACAGGCCTTATTGGCTATTCAGGGCTCAGGGCATGAACAATGGGATTGCATGGAATAA TTTATTATTTTTAACTGTAGGGGATAACACACGGGGAACTAACCTTAGTATTAGTGTAGCTGCAGA TGGAAACGCATTGTCAGAGTATGATACTGGCAAATTTAACCTATACCATAGGCATATGGAAGAATA TAAGCTAGCATTTATATTGGAGCTGTGCTCTGTTGAGATTACTGCACAAACACTGTCACATCTGCA AGGACTGATGCCCTCTGTGCTACAAAACTGGGAAATCGGGGTGCAACCTCCTGCTTCTTCTATTT

TAGAAGATACTTATAGGTACATAGAGTCTCCTGCAACTAAATGTGCAAGTAATGTTATACCACCCA
AAGAAGACCCTTATGCAGGGCTTAAGTTTTGGAGCATAGACTTAAAAGAAAAGCTGTCTTTGGACT
TAGACCAATTTCCCTTGGGAAGAAGATTCTTAGCTCAGCAAGGGGCAGGATGTTCAACTGTGAGA
AAGAGAGCTGTTGCAACCAGAAATTCCAGTAAGCCTGCAAAAAGAAAAAAAATCAAAGCTTAA-3’).

Annex B – Calibration curve used for protein quantification:

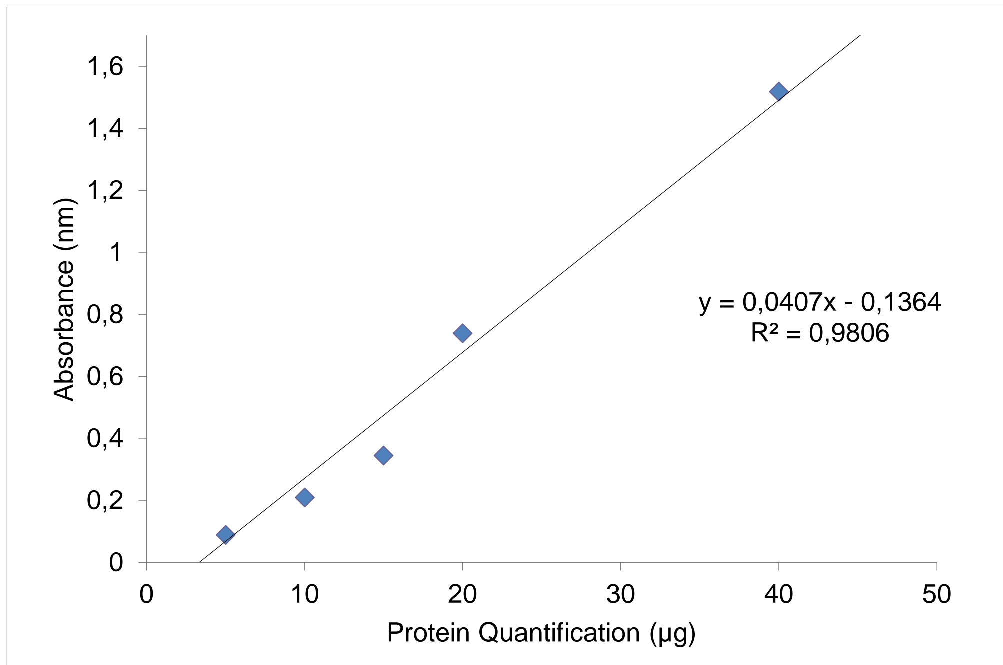

Figure 24 - Calibration curve used for protein quantification.

Annex C – DNA Molecular Ladders:

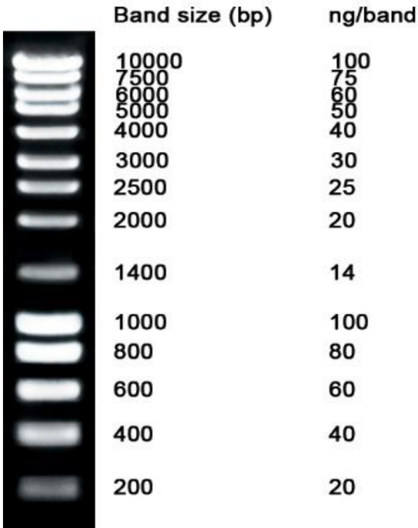

Figure 25 – NZYDNA Ladder III (NZYTech®).

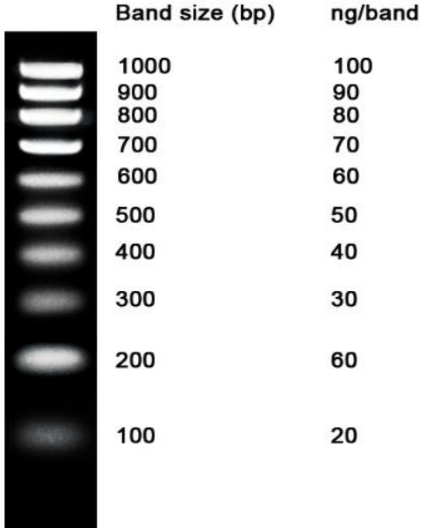

Figure 26 – NZYDNA Ladder V (NZYTech®).

Annex D – Protein Ladder:

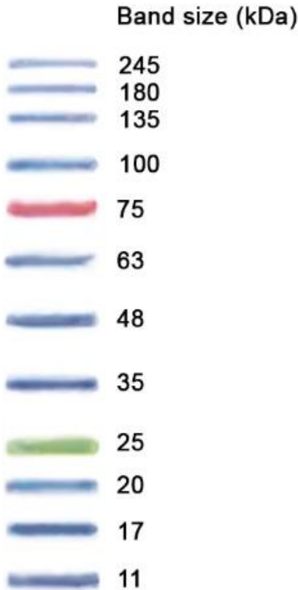

Figure 27 - NZYColour Protein Marker II (NZYTech®).