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

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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.

1. INTRODUCTION

Papillomaviruses (PVs) are a group of small DNA viruses that can cause benign infections in vertebrates [1]. 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) [2], [3]. The inclusion of the different species inside the several genera is based on the analysis of the phylogenetic relativity, biological properties and nucleotide sequence similarity of the L1 gene ORF (open reading frame), because it is the most conserved gene in the PV genome [4].

BPV-1 and BPV-2 are closely related genotypes, sharing 92% homology in their L1 protein sequence [5]. These two BPV types have the ability to infect both epithelial and mesenchymal cells, and 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 [6], [7]. BPV-2 can also infect the urinary bladder epithelium and esophagus, wherein no virions are produced. The concomitant ingestion of immunosuppressants by the BPV-2 infected animal can lead to the development of malignant carcinomas in the urinary bladder [5]. 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. Furthermore, some insects may be vectors through which the virus may be transmitted [8].

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 [9]. Inside this structure, 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 elements essential for the viral DNA replication and transcription – being the other two regions identified as early (E) and late (L) genes, in accordance to the host's cell differentiation stage where they are expressed [2]. 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 [2], [9]–[11]. The E5, E6 and E7 proteins belong to the viral oncoproteins

group, and this group of oncoproteins have the ability to stimulate cell proliferation and survival, and modulate the differentiation of keratinocytes, being E5 the major transforming oncoprotein in BPV-2 [12]. Nevertheless, E6 and E7 proteins are as well crucial in cell transformation [13], [14].

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 [2], [15]. The epithelium differentiation triggers the L1 and L2 genes expression and increase of the viral genome replication, leading to the assembly of viral particles in the nucleus [16]. The L1 protein has the ability to self-assemble in the absence of L2, in a spontaneously way, into virus-like particles (VLPs) [17]. VLPs are antigenically highly similar to PV virions, having the ability to induce high titers of type-specific neutralizing antibodies [5].

It has been proven that vaccines based on VLPs are safe and effective in the prevention of infections of several pathogenic viruses [18]. Furthermore, 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 [19].

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 [10]. Additionally, expression and characterization of oncoproteins, and structural proteins, have the potential to offer important biotechnological products, from antibodies to potential vaccines [20]. In this experimental work, it was made an effort to clone and express two early genes – E6 and E7 – and one late gene – L1.

2. MATERIAL AND METHODS

2.1 *E6, E7 and L1 Genes Amplification.*

For E6 (414bp), E7 (384bp) and L1 (1494bp) amplifications, the following specific primers were designed: E6 sense primer, 5'-CTA**GC TAGC**ATGGACCTGCAAAGTTTTTCC-3'; and E6 antisense primer, 5'-CCG**CTCGAG**TGGGTAGTTG GACCTTGAACC-3', E7 sense primer, 5'-CTA**GCTA GC**ATGGTTCAAGGTCCAACTACC-3'; and E7 antisense primer, 5'-CCG**CTCGAG**TCGTTTGCCAT GACGCTC-3', L1 sense primer, 5'-CG**GAATTC**ATG GCGTTGTGGCAACAAGGCCAG-3'; and L1 antisense primer, 5'-CCG**CTCGAG**AGCTTTGATTT TTTTTCTTTTTGCAGGC-3'. Primers were designed to include in the 5' prime end different restriction sites, allowing a directional cloning into the plasmid expression vector pET-24a (Novagen®). Each gene sequence was analyzed for restriction mapping, using the CLC Main Workbench Program (CLCBio, Qiagen®). For E6 and E7 genes, the *NheI*/*XhoI* pair was chosen and for the L1 the pair *EcoRI*/*XhoI*. Previous to each restriction site a three nucleotide tail was added, according to the NEB-Guide [21], to fulfill the restriction enzyme requirement. The underlined sequences represent the 5' flanking nucleotides (NEB-Guide [21]); the bold sequences represent the restriction endonucleases recognition sites, followed by the specific gene sequences of BPV-2.

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 a DNA sample of a *BPV-2* positive papilloma, collected from a cow.

For E6 and E7 genes amplification the cycling conditions included 10 minutes at 95ºC for an 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; for L1, 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. All amplifications were performed on a Doppio thermal cycler (VWR®).

2.2 *E6, E7 and L1 Cloning.*

The presence of the specific amplicons was confirmed in a 1.5% agarose gel electrophoresis and purified using the kit DNA Clean & Concentrator™-5 (ZYMO RESEARCH®). For directional cloning into the expression plasmid – pET-24a (Novagen®), both plasmid and amplicons were double hydrolyzed with two different 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. The ligation was performed using a Speedy Ligase (NZYTech®), following the manufacturer's instructions. After ligation of E6, E7 and L1 insert sequences within pET-24a, E. coli DH5α competent cells were transformed by heat shock. Positive recombinant clones were selected on LB plates containing kanamycin, and the correct insertion of the E6, E7 and L1 ORFs into the cloning sites was verified by DNA sequencing. The recombinant plasmid DNA

was later used to transform E. coli BL21(DE3)pLysS competent cells, by heat shock. Positive recombinant clones were selected on LB plates containing kanamycin and chloramphenicol, and the correct insertion of the E6, E7 and L1 ORFs into the cloning sites was verified by DNA sequencing.

2.3 *Protein Expression.*

Protein expression of BPV2 E6, E7 and L1 genes was carried out according to the Novagen pET System Manual, and The QIAExpressionist Handbook [22], [23]. Briefly, a 10ml starter culture was grown in LB broth (NZYTech®) with 1µL/mL kanamycin and 1µL/mL chloramphenicol overnight at 37ºC in a Gallenkamp Orbital Incubator at 200 RPM/minute; 3mL were later seeded in 50mL of LB broth (NZYTech®) with the same antibiotics, and was grown at 37ºC in a Gallenkamp Orbital Incubator at 200RPM/minute until the cell density reached OD_{600}=0.5 – 1. To induce E6, E7 and L1 expression IPTG (NZYTech®) was added to the culture, to a final concentration of 1mM. The culture was grown for 4 hours at 37ºC with agitation (200RPM/minute) in a Gallenkamp Orbital Incubator, and 2mL aliquots were collected hourly. The aliquots were centrifuged at 1000G's for 5 minutes, and the pellet and supernatant were separately stored at -20ºC. At the end of the 4 hours incubation period, the culture was centrifuged at 4000G for 20 minutes, and the pellet was stored at -20ºC.

2.4 *SDS-PAGE.*

In order to extract the expressed protein from inclusion bodies, the induced cells from each collection point were digested overnight in 10mM Tris-HCL (pH=8), 1M NaCl, 50mM Urea, 1M β-Mercaptoethanol and 20mM Imidazole. The digested cells were centrifuged for 20 minutes at 13.5RPM. The lysed solution was later purified in a His Spin Trap columns (GE Healthcare®), following the manufacturer's instructions. Each protein elution aliquots were quantified using the Bradford method.
Samples were resuspended in 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®). Posteriorly, denatured samples were run in a 15% polyacrylamide gel and the SDS-PAGE gel was stained with 25mL of BlueSafe (NZYTech®), for protein visualization. A parallel gel was run and transferred to 0.2µm PVDF membrane (invitrogen®), in 1x Transfer buffer (25mM Tris base, 192mM Glycine, 10% Methanol and dH_2O) at 40mA overnight.

2.5 *Immunoblotting.*

PVDF membrane was previously incubated in Blocking buffer (10% (w/v) non-fat dry milk, 1x PBS (SIGMA-ALDRICH®) and 0.05% Tween™-20), during 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 a 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 was used a 4-Chloro-1-naphthol solution (83mM 4-Chloro-1-naphthol, 0.06% H_2O_2 and 96% TBS).

Table 1 – 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 (1:3000)	Anti-Mouse IgG/PO (SIGMA-ALDRICH®) (1:50000)
L1	BPV-2 positive cow serum (1:10)	Anti-Bovine IgG/PO (1:1000)
	Anti-His Mouse IgG (1:3000)	Anti-Mouse IgG/PO (SIGMA-ALDRICH®) (1:50000)

2.6 *ELISA.*

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 1x PBS; blocked with PBS-BSA 3% per well and incubated at 37ºC for 1 hour. The plate was washed five times with 1x PBS per well and the primary antibody was added to each well in duplicates; the plate was incubated at 37ºC for 1 hour. After another washing step, the 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 a substrate solution (ABTS – *2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)*) plus 1.7% H_2O_2 (30%)) was added to each well. The results were obtained by reading the optical density at 405nm at different time points. To read the ELISA plates it was used a SpectraMAX 340PC microplate reader (MOLECULAR DEVICES®). The ELISA results presented were obtained 10 minutes after incubation with the substrate solution for E7, and 20 minute after incubation with the substrate solution for L1.

Table 2 - 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)

3. RESULTS

3.1 *E6, E7 and L1 Genes Amplification and Cloning.*

The E6 gene sequence (414bp) was amplified from a previously cloned recombinant plasmid (pGEM®-T vector) sample. For the E7 (384bp) and L1 (1494bp) genes amplification, DNA samples of BPV-2 positive cows were used. The three genes were successfully amplified. The amplified genes were digested and cloned inside pET-24a, and the recombinant DNA was Sanger sequenced. The E7 and L1 sequencing results showed that the genes specificity and correct orientation. However, in the case of E6, only the sequencing result with the forward sequencing primer was obtained, and it showed a nucleotide insertion immediately after the primer sequence. Although this E6 sequencing result, E6, E7 and L1 recombinant plasmid were further used to transform E. coli BL21(DE3)pLysS competent cells. Posteriorly, the transformed E.coli BL21(DE3)pLysS cells were IPTG induced.

3.2 *SDS-PAGE Analysis.*

The E6 protein (15.8kDa) was not detected, being no image presented.
In regarding to E7 (13.6kDa), a protein band was detected in the four time points (t1; t2; t3; t4) with increasing intensity, which indicates an increasing protein expression in each time points. However, due to the high protein diversity of the whole protein extract, E7 samples were purified in a His Spin Trap columns (GE Healthcare®) and were run in a SDS-PAGE. A protein band of E7 expected molecular weight was detected in the first and second elution steps (Figure 1 – **A**).
For L1, the high protein diversity of the whole protein extract made it difficult to confirm the presence of the protein band. So, as for E7, the samples were purified in a His Spin Trap column (GE Healthcare®) and run in a SDS-PAGE. After purification, a band of the putative L1 protein (55.5kDa) was evidenced, which is highlighted within the red rectangle (Figure 1 – **B**, Left). The same bands are not present in the non-recombinant pET samples, reinforcing the hypothesis of a correct E7 protein expression (Figure 1 – **B**, Right).

3.3 Immunoblotting.

For E7, to confirm the specificity of the 13.6kDa protein band, an immunological detection was performed, using a rabbit Anti-E7 antibody [24], kindly provided by Professor Peter Howley, Harvard Medical School, and an Anti-Rabbit IgG/PO as secondary antibody, which resulted in a positive staining of the corresponding E7 protein band (Figure 2 – **A**). The L1 protein was equally tested by Immunoblotting, using BPV-2 positive cow serum as primary antibody, but no visible bands were detected.
In order to detect the E7 and L1 proteins by their histidine tail, an Anti-His Mouse IgG was used as primary antibody. However, no visible bands were seen, and therefore no image is presented.

3.4 ELISA.

The specificity of the recombinant E7 and L1 proteins was further assessed by ELISA using the rabbit anti-E7 IgG and the BPV-2 positive cow serum, respectively.
The specificity of the anti-E7 IgG was also tested, using an HIV Integrase (Figure 2 – **B**), and only the E7 protein coated wells recorded a significant absorbance signal, confirming the Anti-E7 IgG specificity to the E7 protein. In Figure 3, in the wells coated with the E7 antigen, an expressive absorbance signal is detected from the 1:500 up to

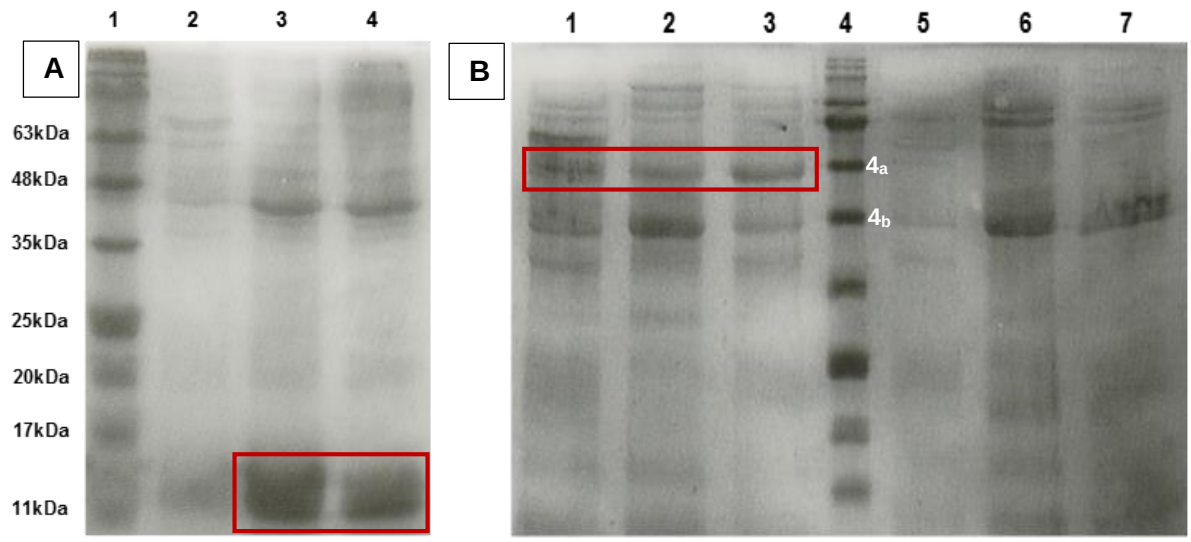

*Figure 1 – **A:** 15% SDS-PAGE of the purified E7 sample. Lane 1: NZYColour Protein Marker II (NZYTech®), Lane 2: Sample from after the first washing step, Lane 3: Samples from the first elution step, Lane 4: Sample from the second elution step. The E7 protein is highlighted within the red rectangle. **B**: 15% SDS-PAGE of the purified L1 (Left) and non-recombinant pET (Right) samples. 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.*

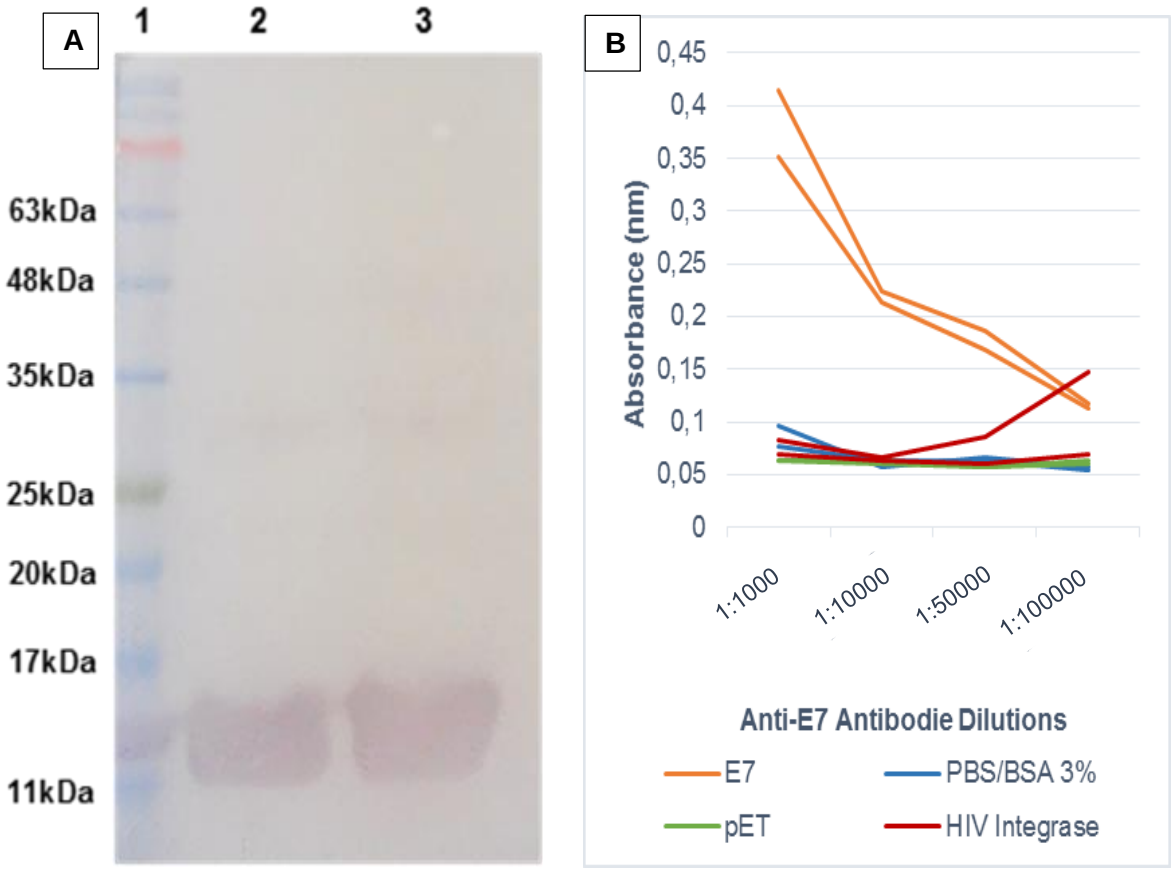

*Figure 2 – **A**: Immunoblotting of E7 protein (13.6kDa) in PVDF membrane (invitrogen®) using Anti-E7 Rabbit IgG as primary antibody and Anti-Rabbit IgG as secondary antibody. Lane 1: NZYColour Protein Marker II (NZYTech®), Lane 2-3: E7 protein. **B**: Diagram of the ELISA results when using an Anti-E7 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.*

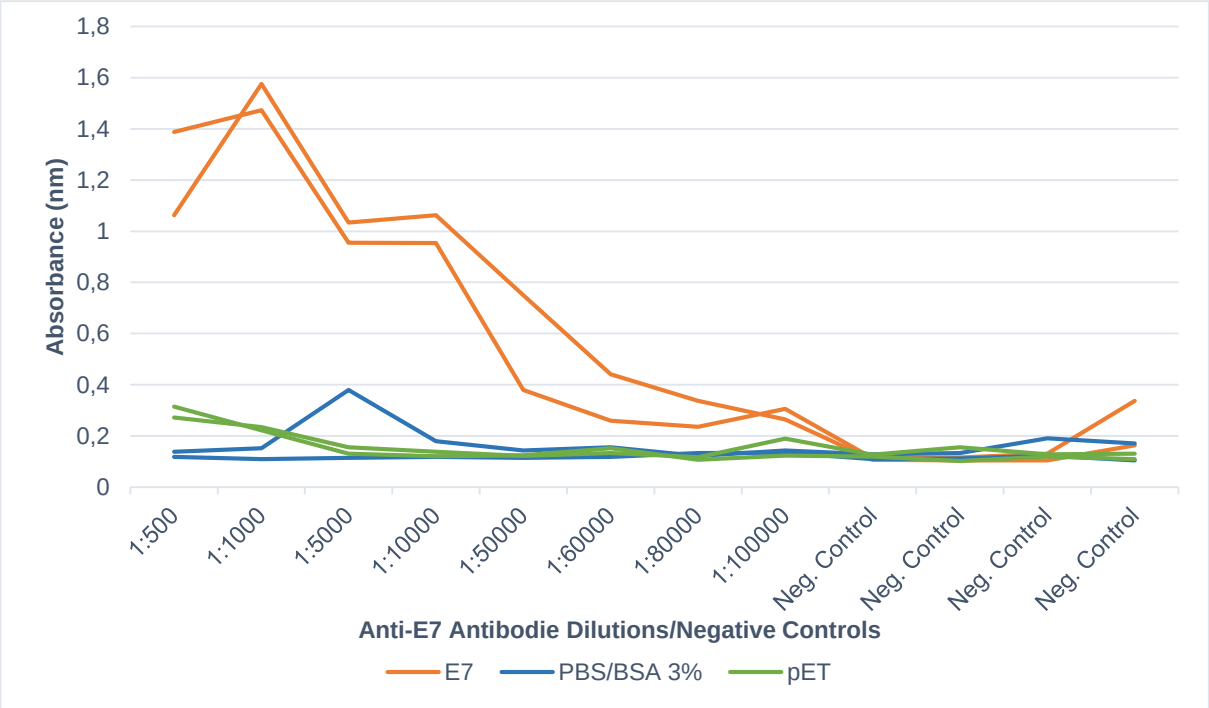

Figure 3 - Diagram of the ELISA results when using an Anti-E7 rabbit IgG as primary antibody (1:500; 1:1000; 1:5000; 1:10000; 1:50000; 1:60000; 1:80000 and 1:100000) and an Anti-Rabbit IgG/PO as secondary antibody. The ELISA was performed in wells coated with the expressed E7 protein, non-recombinant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

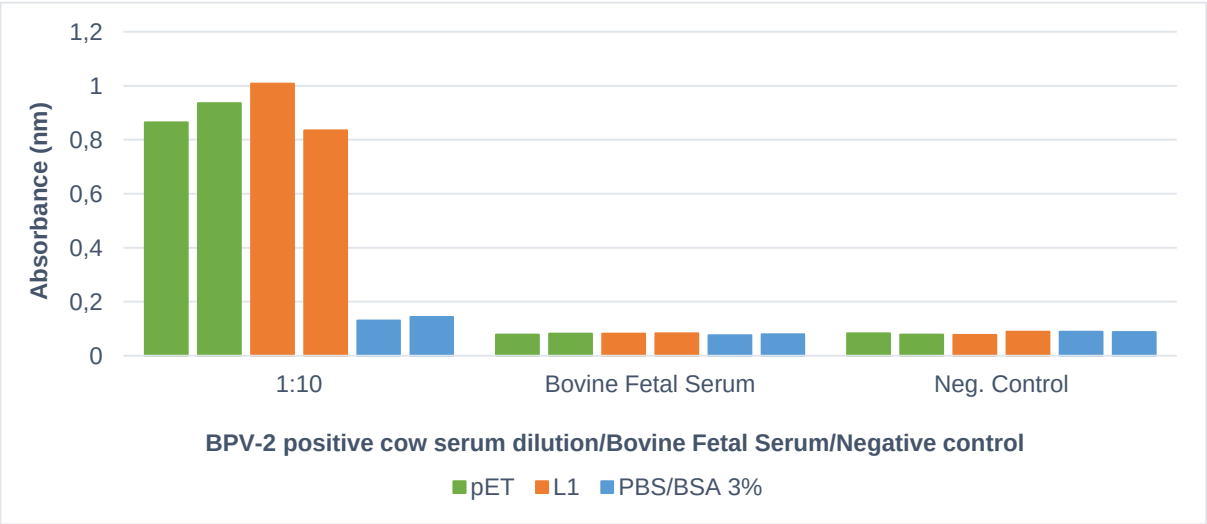

Figure 4 - Representative diagram of the ELISA results using BPV-2 positive cow serum and Bovine Fetal Serum as primary antibodies, and Anti-Bovine IgG as secondary antibody. The ELISA technique was performed in wells coated with the expressed L1 protein, non-recombinant pET and Blocking Buffer (PBS/BSA 3%). For the negative control, 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.

the 1:100000 Anti-E7 rabbit IgG dilution. However, when using a 1:500 dilution of Anti-E7 IgG, the absorbance signal is lower than the one registered at 1:1000 dilution, which can be explained by an inhibition of the reaction at higher concentrations of primary antibody, i.e., prozone effect. Furthermore, at 1:1000 dilution of Anti-E7 IgG, the absorbance signal reaches its maximum (Absorbance≈1.50). In the wells incubated with the secondary antibody (Negative 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, are constantly superior to the signals registered in the control wells (non-recombinant pET and blocking buffer wells). This is an evident proof of the E7

specificity. In L1 ELISA, due to the lack of an Anti-L1 IgG, a BPV-2 positive cow serum was used as primary antibody. The presence of anti-*E. coli* antibodies in the serum could lead to confusing ELISA results. Hence, the BPV-2 positive cow serum was pre-incubated with non-recombinant pET samples, in order to reduce its anti-*E.coli* antibodies concentration. When using a BPV-2 positive cow serum as primary antibody, an approximately similar absorbance value is detected between L1 and the non-recombinant pET. Like 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 as expected, due to the lack of antibodies in this serum, the registered absorbance signals were also extremely low (Figure 4). The similar absorbance signal registered both in L1 and non-recombinant pET do not confirm the putative L1 specificity. However, the strategy used to reduce the anti-*E. coli* antibodies titer in the serum must be seen as inefficient and further improvement would lead to more convincing results.

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.

E6 is one of the oncogenes present in the viral genome. The E6 protein is associated with cell transformation and immortalization [13]. 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.

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 [14], [24]–[26]. 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 [24]. 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.

The L1 protein is the major structural protein that constitutes the PV virion capsid and, together with L2, is essential in the virus infectivity [2]. 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 [17]. HPV is considered as the main causal agent in cervical cancer [27]. 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 [28]. When comparing to live and attenuated vaccines, VLP-based vaccines are considerably safer, which makes them ideal investigation targets nowadays [28].

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 [7], 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 [19], [29]. 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 [12], which makes it an ideal candidate to study and investigate.

The expression of recombinant oncoproteins allows the development of valuable diagnostic tools [20], 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 [24]. 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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