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Short Communication

Development of a DNA-based vaccine strategy against bovine papillomavirus infection, involving the E5 or L2 gene

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ABSTRACT. Papillomaviruses are known to cause tumor lesions, generally benign, in epithelial tissues of diverse organisms; these lesions may progress to cancer under suitable conditions. Bovine papillomavirus (BPV) can cause urinary bladder cancer and cancer of the upper gastrointestinal tract. Furthermore, BPV1 and BPV2 are implicated in the development of tumors in equids. Many studies with animal models clearly demonstrate that DNA vaccines are very effective tools in controlling viral infections, providing strong humoral and cellular immune responses. In this study, we have described the development of two vaccine constructs for the control of diseases caused by BPV. The 1st strategy is prophylactic and is based on the L2 gene; the 2nd is therapeutic and is based on the E5 gene. Vaccine constructs were obtained and evaluated *in vitro* in mammalian cells. The results show the occurrence of E5 and L2 transcription and viral protein production. These results confirm the functionality of the vaccine constructs in mammalian

cells. This is the 1st step in the development of a DNA-based vaccine strategy for the control and/or treatment of diseases caused by BPV.

Key words: Bovine papillomavirus; BPV; DNA vaccine

INTRODUCTION

The cattle industry is one of the mainstays of the Brazilian international agribusiness. However, some diseases are considerably damaging this sector. Among them there are diseases caused by papillomavirus, that is, papillomatosis and cancer. Bovine papillomatosis is characterized by the presence of tumors that occur in the skin, mucous membranes, and some organs. It is an important disease leading to economic depreciation of animals and the deterioration of the appearance of the animal and animal leather. Additional economic damage is caused by genital warts, since they lead to loss of reproductive function in both male and female cattle (Campo, 2003). Besides the benign tumors, there is the possibility, under the action of co-factors, of progression to cancer in the upper gastrointestinal tract or urinary bladder, which usually leads to the death of the animal (Campo, 2006). Currently, there are 13 known different types of bovine papillomavirus (BPV), classified according to biological properties and genome organization (Freitas et al., 2011; Lunardi et al., 2013). Although papillomaviruses are considered strictly species specific, BPV1 and, less frequently, BPV2 are recognized as the most important etiological agents in the development of fibroblastic skin tumors or equine sarcoids, which affect horses, donkeys, and mules (Chambers et al., 2003; Nasir and Campo, 2008). Currently, there is no vaccine or effective treatment for the control of papillomatosis and the related cancers.

Several strategies have been used in the development of different types of vaccine (Kim et al., 2008). DNA vaccines have been considered because they are able to stimulate effective cytotoxic T lymphocyte and antibody responses by delivering foreign antigens to antigen presenting cells that stimulate $CD4^+$ and $CD8^+$ T cells. Naked plasmid DNA vaccines offer a number of advantages when compared to classical vaccines (Gurunathan et al., 2000). They can be produced in a short time, the cost of large-scale production is considerably lower, and the commercial distribution does not require a cold chain because these vaccines are stable at room temperature (Glenting and Wessles, 2005).

E5 protein is the major BPV oncoprotein that acts by disturbing the mechanisms of growth suppression and cell cycle control. Additionally, it interacts with growth factor receptors, activating cellular proliferation (Venuti et al., 2011). L2 protein is the minor viral capsid protein but is essential for papillomavirus infection. It is responsible for binding to a secondary viral receptor, thereby facilitating the exit from the endosome and the delivery of the viral genome to the nucleus (Karanam et al., 2009).

Development of a prophylactic and therapeutic vaccine for BPV infection has become a key issue. This study aimed to construct vaccine vectors based on the BPV1 E5 and L2 genes and to evaluate their potential for expression in mammalian cells.

MATERIAL AND METHODS

The BPV1 E5 gene was codon-optimized for expression in mammalian cells and then synthesized by Epoch Biolabs, Inc. (Fort Bend County, TX, USA). Besides codon optimiza-

tion, it was necessary to neutralize the transforming activity of this gene, since we aimed to construct a DNA vaccine for immunization. Therefore, glutamine 17 was replaced by glycine, as this substitution is known to eliminate the biological activity of the E5 protein (Sparkowski et al., 1994). The L2 gene was amplified by polymerase chain reaction (PCR) from the complete BPV1 genome and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). For both genes, the AU1 epitope was inserted at the N-terminal portion to facilitate immunological detection (Lim et al., 1990). The genes were subcloned into the pCI-neo expression vector (Promega), generating the pCIE5sint and pCIL2 constructs. The presence and orientation of the insert were analyzed by PCR, enzyme digestion, and DNA sequencing.

HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, São Paulo, SP, Brazil), supplemented with 10% fetal bovine serum (Gibco, São Paulo, SP, Brazil), 1% penicillin/streptomycin (Gibco), and 1% L-glutamine (Sigma, São Paulo, SP, Brazil). Cells at approximately 80% confluence were transfected with the pCIE5sint, pCIL2 constructs or the empty pCI-neo vector (negative control) by using Polyfect® transfection reagent (Qiagen, São Paulo, Brazil), following the manufacturer protocol. After 1 h of incubation, the transfection reagent was removed and complete DMEM was added. Cells were incubated for 48 h at 37°C in 5% CO_2. The expression of viral genes and production of viral proteins were then evaluated as described below.

To evaluate the transcription of the E5 and L2 genes, RT-PCR was performed on the transfected HEK 293 cells. Total RNA was extracted using the RNeasy RNA isolation kit (Qiagen). The synthesis of cDNA was performed using the ImProm®-II Reverse Transcription System (Promega), following manufacturer recommendations. To eliminate the possibility of genomic DNA contamination of the RNA samples, the material was treated with DNase I (Promega).

Protein expression was evaluated by Western blot analysis. Protein extracts of the transfected cells were resuspended in denaturing buffer with protease inhibitor (Roche, São Paulo, SP, Brazil) and heated at 95°C for 5 min. The proteins were separated on 12.5 and 15% SDS-PAGE and transferred onto PVDF membranes (Millipore, São Paulo, SP, Brazil). The membrane was blocked with 5% nonfat dry milk, incubated with anti-AU1 monoclonal antibody (Covance, Pricenton, NJ, USA) (1:500) under agitation for 1 h, and washed 3 times with PBS 0.05% Tween 20. The membrane was then incubated for 1 h with anti-mouse IgG HRP conjugated (1:5000) (Jackson ImmunoResearch, West Grove, PA, USA) and washed with PBS-0.05% Tween 20. Protein detection was performed using the ECL chemiluminescent kit (GE Healthcare, São Paulo, SP, Brazil).

Because E5 is a membrane protein, it was necessary to perform an immunoprecipitation assay before the Western blot analysis. Therefore, the protein extract from cells transfected with the pCIE5sint construct was incubated for 5 min with radioimmunoprecipitation (RIP) buffer and protease inhibitor, homogenized, and centrifuged at 8000 rpm for 5 min. The supernatant then was incubated with anti-AU1 antibody (1:150) for 1 h at 4°C under agitation. A suspension of protein A-Sepharose CL-4B (Invitrogen) was then added and the mixture incubated for 1 h at 4°C with constant rotation. The immunocomplex was washed four times with cold RIP buffer, resuspended in denaturing buffer, and heated for 5 min at 95°C.

RESULTS AND DISCUSSION

The production of an effective, safe, low-cost vaccine should lead to a large reduction in the incidence and mortality of animals infected with BPV. This virus is also an excellent

model for human papillomavirus vaccination studies. For these reasons, BPV E5 and L2 proteins have been considered strong candidates for DNA vaccines against bovine papillomatosis. In this study, the viral genes were cloned into an expression vector and evaluated for *in vitro* gene transcription and consequent production of viral proteins.

Evaluation of viral gene transcription was performed by RT-PCR in HEK 293 cells transfected with pCIE5sint or pCIL2 constructs. As negative controls, we used cells transfected with empty pCI-neo vector and non-transfected cells. In this assay, we observed amplification of a specific 175-bp fragment related to the E5 gene and a 500-bp internal fragment of the L2 gene (Figure 1). There were no amplified products from samples where the reverse transcriptase enzyme was omitted, indicating there was no contamination with genomic DNA.

Protein extracts of the transfected cells were assessed for the presence of the viral proteins by Western blot. The assay detected bands of approximately the expected sizes for BPV1 E5 and L2 proteins (10 and 72 kDa, respectively), only in cells transfected with the pCIE5sint or pCIL2 constructs (Figure 2). It is noteworthy that the E5 protein was detected only after an immunoprecipitation step. This step solubilizes biomembranes, releasing the E5 protein from the cell membranes, resulting in a higher concentration of antigen in the extract (Disbrow et al., 2003).

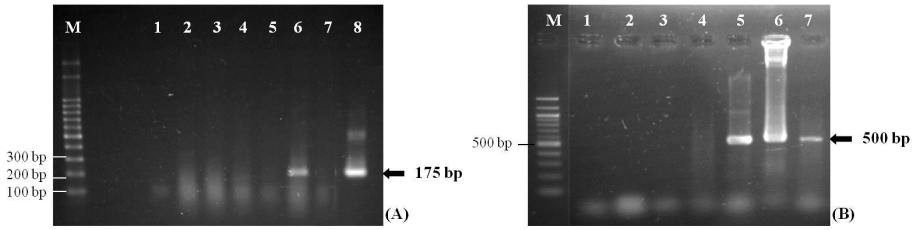

Figure 1. RT-PCR detection of the viral gene transcription in the transfected cells using the gene-specific primers. **A.** Analysis of E5 gene transcription showing a fragment of 175 bp. *Lane M* = 100-bp DNA ladder (Invitrogen); *lane 1* = control reaction (without DNA); *lanes 2*, *4* and *6* = respectively, only HEK 293 cells, cells transfected with the empty pCI-neo vector and cells transfected with pCIE5sint; *lanes 3*, *5* and *7* = respective control experiment of genomic DNA contamination, in which the reaction was performed without the reverse transcriptase enzyme; *lane 8* = positive control reaction. **B.** Analysis of L2 gene transcription showing the amplification a 500-bp fragment on the central portion of the L2 gene. *Lane M* = 100-bp DNA ladder (Invitrogen); *lanes 1* and *2* = only HEK 293 cells; *lanes 3* and *4* = cells transfected with the empty pCI-neo vector; *lanes 5* and *6* = cells transfected with pCIL2; *lane 7* = positive control reaction.

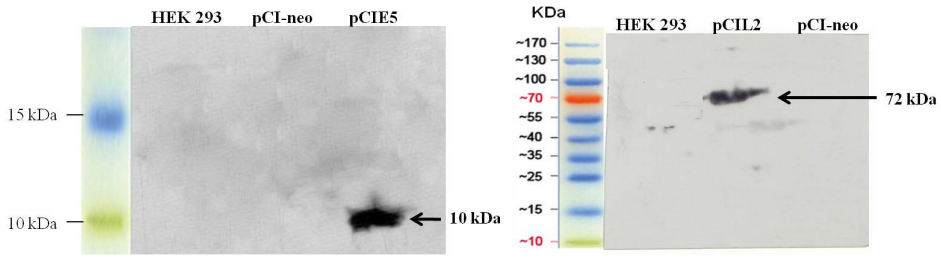

Figure 2. Western blot detection of E5 and L2 proteins produced in transfected cells. The proteins from only HEK 293 cells, cells transfected with the empty pCI-neo vector and cells transfected with pCIE5sint and pCIL2 were analyzed using antibody anti-AU1. A band of approximately 10 and 72 kDa (expected sizes for BPV1 E5 and L2 proteins) was detected only in the cells transfected with pCIE5sint and pCIL2, respectively. The PageRuler Prestained Protein Ladder (Thermo Scientific) was used.

Some studies have already shown the immunogenic potential of the L2 protein against papillomavirus infections, identifying neutralizing and tumor rejection epitopes. However, the main advantage of a vaccine using the L2 gene is the potential to induce a broad spectrum of neutralizing antibodies, possibly with immune cross-reactivity, i.e., L2 can confer immune protection against a range of papillomaviruses, including HPV (Karanam et al., 2009; Jagu et al., 2011). In the case of E5, the possibility of using the gene as a therapeutic vaccine against papillomavirus is still underexplored. The BPV E5 protein is involved in cell transformation; its expression occurs both at the initiation of and during viral infection, modifying the cellular response to growth factors and blocking the expression of the major histocompatibility complex on the cell surface, making this gene attractive for use as a DNA vaccine (Borzacchiello and Roperto, 2008; Venuti et al., 2011).

The results presented here show *in vitro* transcription and translation of the E5 and L2 viral proteins, confirming the functionality of the vaccine constructs in mammalian cells. This is the 1st step in the development of a DNA-based vaccine strategy for the control and/or treatment of diseases caused by BPV.

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REFERENCES

Borzacchiello G and Roperto F (2008). Bovine papillomaviruses, papillomas and cancer in cattle. *Vet. Res.* 39: 45.

Campo MS (2003). Papillomavirus and disease in humans and animals. *Vet. Comp. Oncol.* 1: 3-14.

Campo MS (2006). Bovine Papillomavirus: Old System, New Lessons? In: Papillomavirus Biology: from Natural History to Vaccine and Beyond (Campo MS, ed.). Caister Academic Press, Wymondham.

Chambers G, Ellsmore VA, O'Brien PM, Reid SW, et al. (2003). Association of bovine papillomavirus with the equine sarcoid. *J. Gen. Virol.* 84: 1055-1062.

Disbrow GL, Sunitha I, Baker CC, Hanover J, et al. (2003). Codon optimization of the HPV-16 E5 gene enhances protein expression. *Virology* 311: 105-114.

Freitas AC, Silva MAR, Jesus ALS, Mariz FC, et al. (2011). Recent insights into Bovine papillomavirus. *Afr. J. Microbiol. Res.* 5: 6004-6012.

Glenting J and Wessels S (2005). Ensuring safety of DNA vaccines. *Microb. Cell Fact.* 4: 26.

Gurunathan S, Klinman DM and Seder RA (2000). DNA vaccines: immunology, application, and optimization. *Annu. Rev. Immunol.* 18: 927-974.

Jagu S, Malandro N, Kwak K, Yuan H, et al. (2011). A multimeric L2 vaccine for prevention of animal papillomavirus infections. *Virology* 420: 43-50.

Karanam B, Jagu S, Huh WK and Roden RB (2009). Developing vaccines against minor capsid antigen L2 to prevent papillomavirus infection. *Immunol. Cell Biol.* 87: 287-299.

Kim D, Gambhira R, Karanam B, Monie A, et al. (2008). Generation and characterization of a preventive and therapeutic HPV DNA vaccine. *Vaccine* 26: 351-360.

Lim PS, Jenson AB, Cowsert L, Nakai Y, et al. (1990). Distribution and specific identification of papillomavirus major capsid protein epitopes by immunocytochemistry and epitope scanning of synthetic peptides. *J. Infect. Dis.* 162: 1263-1269.

Lunardi M, Alfieri AA, Otonel RA, de Alcantara BK, et al. (2013). Genetic characterization of a novel bovine papillomavirus member of the *Deltapapillomavirus* genus. *Vet. Microbiol.* 162: 207-213.

Nasir L and Campo MS (2008). Bovine papillomaviruses: their role in the aetiology of cutaneous tumours of bovids and

equids. *Vet. Dermatol.* 19: 243-254.
Sparkowski J, Anders J and Schlegel R (1994). Mutation of the bovine papillomavirus E5 oncoprotein at amino acid 17 generates both high- and low-transforming variants. *J. Virol.* 68: 6120-6123.
Venuti A, Paolini F, Nasir L, Corteggio A, et al. (2011). Papillomavirus E5: the smallest oncoprotein with many functions. *Mol. Cancer* 10: 140.