



DNA and Non-Replicating Human Adenovirus Vectors Expressing VP2 Protein of Infectious Bursal Disease Virus Induce Humoral Immunity in the Murine Model

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Abstract

An eukaryotic expression Plasmid (pXL) and a replication-defective human adenovirus serotype 5 (Ad5) carrying the coding sequence of mature VP2 protein from Infectious Bursal Disease Virus (IBDV) were constructed and the evaluation of their immunogenicity was carried out in the murine model. These novel recombinant vectors were able to express VP2 protein in cell cultures and to induce IBDV-seroneutralizing antibodies in mice vaccinated with heterologous prime-boost regimen. To our knowledge, this is the first report on the obtaining of a recombinant Ad5 vector expressing VP2 protein of IBDV. Other experiments will be performed to evaluate its usefulness in the chicken, natural host of IBDV.

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Abbreviations: pXL and pXL-VP2: Eukaryotic Expression Plasmid Empty and Encoding the Sequence of IBDV-VP2 Mature Protein, respectively; Ad5-GFP and Ad5-VP2: Replication-Defective Human Adenovirus Serotype 5 (Ad5) Expressing the Green Fluorescent Protein and the IBDV-VP2 Mature Protein Respectively; D78: Live Commercial IBDV Vaccine (Strain D78).

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Short communication

Infectious Bursal Disease (IBD) is an acute and highly contagious disease that causes immunosuppression, and in some cases mortality, in young chickens, generating significant economic losses in the poultry industry worldwide. Its etiologic agent, Infectious Bursal Disease Virus (IBDV) infects B lymphocytes that mature in the bursa of Fabricius [1]. The icosahedral IBDV capsid is formed by the viral mature protein 2 (VP2) as the only component [2]. This protein has at least three independent conformational epitopes responsible for the induction of neutralizing antibodies in the host [2,3].

Nowadays, IBD is mainly controlled by administration of vaccines based on attenuated viral strains that although induce a neutralizing antibody response, their efficacy depend on both the degree of attenuation and the level of maternal antibodies present in the birds at the time of vaccination. Thus, the less attenuated strains (called "hot vaccines"), able to overcome the passive immunity, induce a moderate atrophy of the bursa which causes immunosuppression interfering with the immunization of other vaccines and/or favoring infections with opportunistic pathogens [2]. Other vaccines use in poultry industry are an immune complex (an IBDV strain plus an anti-IBDV hyperimmune serum [1,2]), and a cell-associated recombinant HVT to express IBDV-VP2 protein *in vivo* [3]. These vaccines are not neutralized by maternal anti-IBDV antibodies, however, they have higher cost due to the addition of anti-serum or to the requirements of liquid nitrogen to maintain the viability of infected cells [4,5]. In this context, the poultry industry requires the development of new immunogens that are safe, effective, stable (physically and genetically), easy to apply and able to overcome the level of pre-existing maternal antibodies. In the last years DNA vaccines and replication-defective human Adenoviruses Serotype-5 (Ad5) have surged as promising vectors for obtaining new immunogens in poultry.

This report describes the construction and characterization of a recombinant eukaryotic expression plasmid (DNA vaccine) and an Ad5 to express *in vivo* the mature VP2 protein of IBDV and the evaluation of their immunogenicity in the murine model. First, the sequence encoding mature VP2 protein of the IBDV (here denominated *vp2 gene*) was obtained. For this purpose, QM7 cells (ATCC[®] CRL1962[™]) were infected with the commercial vaccine NOBILIS[®]-Gumboro D78 and total RNA was extracted using Trizol reagent (Invitrogen[™]) according to manufacturer's instructions. Then, the *vp2 gene* (from 132 to 1454 nt of Segment A, NCBI accession number EU162087) was amplified by RT-PCR using the OneStep RT-PCR kit (QIAGEN[®]), specific primers (VP2 Forward 5'GCTAGCATGACAAACCTGCAAG3' and VP2 Reverse 5'GCGGCCGCTTATGTCCTGC3', with underlined sequences for recognition of the restriction enzymes *NheI* and *NotI* and translation initiation and termination codons in italics, respectively) and the total RNA as template. Later, *vp2 gene* was cloned into pGEM[®]-T-Easy vector (Promega) and the correct nucleotide sequence of *vp2 gene* in pGEMT-VP2 was verified by DNA sequencing service of Macrogen, South Korea. To build a recombinant eukaryotic expression plasmid, the *vp2 gene* was excised from pGEMT-VP2 with *NheI* and *NotI* restriction enzymes and subcloned in the same sites of pXL vector (this plasmid was obtained by modifying the promoter of the commercial vector pCAGGS (NovoPro), and was gently provided by Dr. Saelens from University of Ghent, Belgium). The construct obtained, named pXL-VP2, was sequenced (Macrogen service) to confirm the identity and correct orientation of the insert. On

the other hand, a recombinant Ad5 to express VP2 protein was obtained using the ViraPower[™] Adenoviral Expression System (Invitrogen) following the manufacturer's recommendations. This kit provides the plasmid pAd-CMV/V5-DEST which has the full-length adenoviral genome except that the regions encoding the E1 and E3 proteins are replaced by Cytomegalovirus (CMV) immediate-early promoter followed by *attL* sequences to clone the gene of interest by homologous recombination with a shuttle or donor plasmid. Thus, the *vp2 gene* was released from pGEMT-VP2 by digestion with *EcoRI* enzyme and subcloned in the polylinker (which is flanked by *attL1* and *attL2* sequences) of the shuttle plasmid Gateway[™] pENTR[™]-4 (Invitrogen). Some clones of pENTR-VP2 were sequenced and a plasmid with the required *vp2 gene* orientation was selected. Then, the final construct of approximately 36 kpb, named pAd-VP2, was obtained by homologous recombination *in vitro* between the *attL* sites of pENTR-VP2 and the backbone vector pAd-CMV/V5-DEST. The integrity of the foreign gene in pAd-VP2 was confirmed by sequencing. To obtain recombinant Ad5 particles (rAd5), the plasmid pAd-VP2 was linearized by digesting with restriction enzyme *PacI* (which allows recombinant adenoviral genome to expose the inverted terminal repeat sequences) and transfected into HEK293A cells (Invitrogen[™]). This cell line constitutively expresses the E1 protein supplementing *in trans* to the defective vector to generate recombinant adenovirus particles, Ad5-VP2. In order to evaluate the expression of VP2 protein from both pXL-VP2 and Ad5-VP2 vectors, Western blot (Wb) assays were performed. Briefly, HEK293T or HEK293A cells were transfected or infected with pXL-VP2 or Ad5-VP2, respectively. Cells were harvested 24 and 48 h after and resuspended in Laemmli's sample buffer. The protein extracts were resolved in SDS-PAGE gels (12%) and transferred to nitrocellulose membranes. The presence of recombinant VP2 protein was analyzed using polyclonal anti-VP2 antibodies, provided by Dr. J.F. Rodríguez (Centro Nacional de Biotecnología, Madrid, Spain). As shown in the (Figure 1A), the expression of VP2 protein, with molecular weight of 37 kDa, was only detected at 24 and 48h in protein extracts from pXL-VP2 transfected cells. Conversely, VP2 expression was not evident in infected cells with Ad5-VP2 (data not shown). In order to confirm the expression of the heterologous protein by Ad5-VP2, an alternative methodology was used to prepare the samples. Thus, HEK293A cells were infected with a vaccinia virus that expresses the bacteriophage T7 RNA polymerase (V-T7) and two hours later transfected with pAd-VP2 as this recombinant adenoviral plasmid also possess a T7 promoter sequence upstream of the foreign gene. Monolayers were collected 48 h after and processed as described before. In this way, the expression of the VP2 protein was evidenced in infected and transfected cells with V-T7 and pAd-VP2, respectively (Figure 1B). In order to study the genetic stability of Ad5-VP2, 10 blind passages in HEK293A at multiplicity of infection (moi) of 0.1 were performed. In the last viral stock produced, the presence of *vp2 gene* and VP2 protein expression were confirmed by PCR and Wb assays, respectively (data not shown). Due to a very small proportion of Replication-Competent Adenovirus (RCA) can be generated by recombination between rAd5 vector and the E1 region present in the 293 cell genomes, the presence of the RCA was analyzed. Thus, the Ad5-VP2 stock was subjected to 5 serial passages to high moi (50) in permissive cells (A549 ATCC[®] CCL-185). Cytopathic effect was not observed in A549 monolayers, indicating the absence of contamination with RCA. Finally, the immunogenicity of pXL-VP2 and Ad5-VP2 was assessed in the murine model applying a heterologous prime-boost scheme.

Female BALB/c mice of 7 weeks of age, provided by the biotery of Pablo Cassara Laboratory S.R.L., Argentina; were used. The experiment was carried out in compliance with international legal and institutional guidelines. Groups of five animals were inoculated by intramuscular route with 100µg of pXL-VP2 or pXL (empty plasmid) and, 21 days latter were boosted with 5×10^8 UFP of Ad5-VP2 or Ad5-GFP (rAd5 vector expressing the green fluorescent protein previously obtained in our laboratory [6]), respectively. Another group (positive control) was intraperitoneally immunized with two dose of D78 live vaccine (2.7×10^3 PFU/dose). At days 0 (pre immune), 20 and 41, blood samples were collected and the sera were separated. Neutralizing antibodies to IBDV were analyzed with a varying serum levels-constant virus concentration test as previously described [7]. As expected, neither the pre immune status samples nor sera of mice vaccinated with pXL and Ad5-GFP showed IBDV-specific antibodies (titer <2). Conversely, the presence of IBDV seroneutralizing antibodies was evidenced in samples from animals immunized with pXL-VP2, Ad5-VP2 or D78 vaccine with titers ranging from 64 to 1024 (Table 1).

Vectors based on DNA and Ad5 have several advantageous features to development new vaccine candidates in poultry. These vectors carry the gene of interest cloned for its expression *in vivo*. The *de novo* synthesis of the foreign protein in the vaccinated animals would allow: i) overcoming the interference with Maternally Derived Antibodies (MDA) present in the offspring and ii) to induce specific cellular and humoral immune responses [8,9]. Besides, specific immunity against Ad5 has not been demonstrated in chickens therefore interference with anti-vector antibodies is highly unlikely [9]. Others advantages of rAd5 and DNA vaccines are their simplicity and speed of production, biological stability and biosecurity (infectious agents are not introduced in the injected animals or in the environment). Also, the use of these vectors as immunogens in poultry would allow Distinguishing Infected from Vaccinated Animals (DIVA) [8,9]. Finally, these recombinant immunogens can be administered by mass vaccination methods used in the poultry industry (*in ovo*, subcutaneous injection or oral route in the case of rAd-5) [8,9]. Several reports describe the immunization of chickens with recombinant plasmids encoding VP2 protein. However, in all cases repeated vaccinations (three or four) with those plasmids were necessary to reach adequate protection against IBDV in challenge trials [10-12]. On the other hand, although rAd-5 expressing protective antigens of avian influenza, Marek's disease and infectious bronchitis viruses were successfully evaluated as immunogens for chickens (reviewed in [13]), rAd5 coding the IBDV VP2 protein had not been obtained until now. Based on this background, an eukaryotic expression plasmid and a replication-defective Ad5 encoding IBDV VP2 protein were obtained. First, the expression of the heterologous protein was confirmed in Wb assays. Then, the immunogenicity of both vectors was demonstrated in the murine model by applying a heterologous vaccination regimen. Mice were priming with the DNA vaccine and boosting with the recombinant viral vector because this strategy has been described to be the most effective in heterologous schemes (reviewed in [8]). IBDV neutralizing antibodies were induced by both pXL-VP2 and Ad5-VP2 immunogens. Due to neutralizing VP2 epitopes are discontinuous; the presence of IBDV neutralizing antibodies in the sera of vaccinated mice indicates the appropriate folding of this protein *in vivo*. In murine model, the antibodies titer of the group primed with pXL-VP2 increased after booster with Ad5-VP2 to higher level than that induced

with two doses of the commercial D78 live vaccine (Table 1). These preliminary results encourage the evaluation of the immunogenicity and efficacy of the new vaccine candidates (pXL-VP2 and Ad5-VP2) in the chicken, natural host of IBDV. To our knowledge, this is the first report that describes the obtaining and evaluation of an Ad5 expressing VP2 protein as a vaccine candidate to control of IBDV.

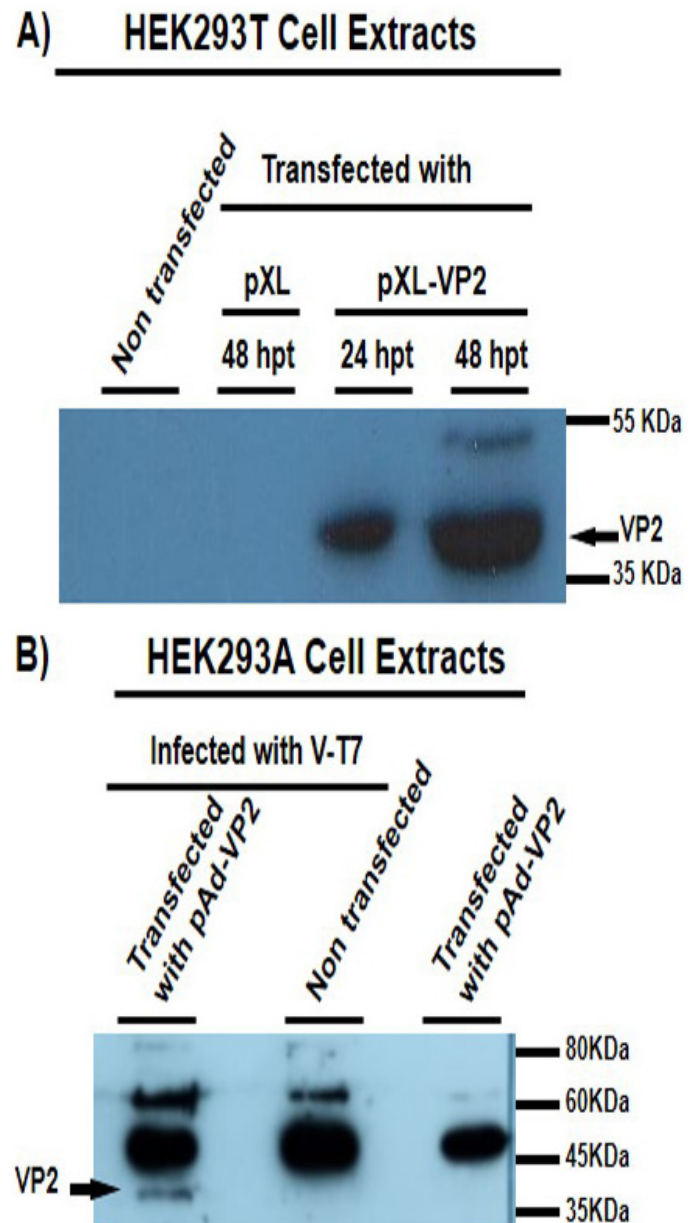


Figure 1: Expression of mature Viral Protein 2 (VP2) from recombinant vectors in Western blot assays.

A) Extracts of HEK293T cells transfected with pXL-VP2 (eukaryotic expression plasmid pXL encoding the sequence of IBDV-VP2 mature protein) and harvested at 24 or 48 h post tranfection (hpt). As controls, extracts of cells transfected with pXL or non transfected.

B) Extracts of HEK293A cells infected with VT-7 (vaccinia virus that expresses the bacteriophage T7 RNA polymerase), transfected with pAd-VP2 (recombinant plasmid containing the adenovirus genome with the coding sequence of VP2 mature protein) and harvested 48 h after. As controls, extracts of cells only infected with V-T7 or transfected with pAd-VP2. In both panel A) and B) the sizes of the marker proteins are on the right and the reactive bands corresponding to VP2 protein are indicated with arrows.

Table 1: Titers of IBDV-neutralizing antibodies in sera of vaccinated mice.

Group type	Immunizations Prime//Boost	Titer Post Prime	Titer Post Boost
Negative control	pXL//Ad5-GFP	<2	<2
Positive control	D78//D78	64	256
Experimental	pXL-VP2//Ad5-VP2	64	1024

Seroneutralizing titers of pools of sera were expressed as the reciprocal of the last dilution that completely neutralized 100 TCID₅₀ (50% tissue culture infective doses) units of IBDV strain D78.

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