

Short Communication

Canarypox virus expressing infectious bursal disease VP2 protein as immunogen for chickens

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Abstract

Canarypox viruses (CNPV) carrying the coding sequence of VP2 protein from infectious bursal disease virus (IBDV) were obtained. These viruses were able to express VP2 protein *in vitro* and to induce IBDV-neutralizing antibodies when inoculated in specific pathogen-free chickens demonstrating that CNPV platform is usefulness to develop immunogens for chickens.

Key words: canarypox virus, viral vector, IBDV, VP2 protein.

Canarypox virus (CNPV), a member of the avipoxvirus genus, has been successfully evaluated as recombinant expression vectors for vaccines development. In this sense, the CNPV platform has been used to give rise protective immune response against various pathogens in different mammalian species (reviewed in Poulet *et al.*, 2007; Brun *et al.*, 2008). Moreover, some of them have reached the market as licensed products commercialized by Merial (a Sanofi Pasteur Company).

Based on the fact that no reports have been published using this viral vector to prevent diseases in chickens, we decided to obtain and evaluate recombinant CNPV as immunogen for that species selecting VP2 protein from infectious bursal disease virus (IBDV) as a model antigen. IBDV is the etiologic agent of infectious bursal disease (IBD), a highly contagious and severe disease that causes significant economic losses in the poultry industry worldwide. IBDV replicates primarily in B lymphocyte precursors developing within the bursa of Fabricius (Müller *et al.*, 2003). Depending on the virulence of IBDV strain and the age of the infected chickens, IBD is manifested in two ways. In some cases occur with clinical symptoms and may cause up to 20% or more mortality in chickens 3 weeks of age and older. Otherwise, and more important, is evidenced by a severe, prolonged immunosuppression of chickens infected

at an early age (Etteradossi and Saif, 2008). The VP2 mature protein (of 441 residues) is the only structural component of the IBDV icosahedral capsid, and thus responsible to the interaction with host cell receptors. VP2 constitutes the major host-protective antigen as it contains conformation-dependent epitopes responsible for eliciting neutralizing antibodies (Müller *et al.*, 2003).

In this work, we obtained recombinant canarypox viruses that express IBDV VP2 protein and showed their immunogenicity when inoculated in specific pathogen-free (SPF) chickens.

The first step was the construction of the transference vector (named TV-CN048-VP2) carrying foreign genes flanked by viral regions (position 57,247, inside CNPV046, to 59,528, inside CNPV048, (Tulman *et al.*, 2004)) which serve as points of recombination with CNPV genome. The selected gene was the coding sequence for VP2 mature protein from the Argentinean IBDV isolate LD-847-04, serotype I (*vp2 gene*, NCBI accession No. JF965438). This nucleotide sequence was obtained as described before (Zanetti *et al.*, 2012a) and subcloned into TV-CN048-GUS plasmid which was previously designed and built in our laboratory (Zanetti *et al.*, 2012b). The final construction was the transference vector (8,452 bp, Figure 1A), which carried *vp2 gene* downstream of the poxviral synthetic

early/late promoter (pE/L) and the expression cassette for the GUS enzyme (*uidA* gene under regulation of vaccinia virus H6 gene promoter), both flanked by CNPV genomic sequences.

The *vp2* gene identity was confirmed by DNA sequencing using an ABI PRISM 3130 genetic analyzer (Applied Biosystem, Japan). Then, the final plasmid TV-CN048-VP2 was transfected, by Lipofectin™ (Invitrogen, Carlsbad, CA, USA), into SPF primary chicken embryo fibroblasts (CEFs) previously infected with CNPV (Abbatista95 vaccine strain provided by LaDiPreVet laboratory, La Plata, Argentina). Recombinant viral clones forming GUS positive plaques were visualized with X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, Inalco, Italy) overlay. After five rounds of purification, 100% of blue plaques were visualized and CN048-VP2 viruses were amplified and molecularly characterized. Firstly, total DNA

extracted from infected CEFs was analysed by Southern Blot (Ferrer *et al.*, 2011). The purity of recombinant viruses (absence of wild type genome) and the insertion of the VP2 expression cassette were confirmed by hybridization with ³²P-labelled CNPV048 probe (genomic positions 58,948 to 59,528) or *vp2* gene probe, respectively (Figure 1B). Thereafter, the expression of VP2 protein from recombinant viruses was evaluated by Indirect Immunofluorescence (IIF) and Western blot (WB) assays in DF1 (ATCC, CRL-12203) infected cells as previously described (Zanetti *et al.*, 2012a). The expression of VP2 protein (green fluorescence) was clearly observed in the cytoplasm of CN048-VP2 infected cells but no fluorescence was evidenced in CNPV-infected cells (Figure 1C). Besides, a band of approximately 37 kDa was only detected by WB in DF1 cells infected with the recombinant CN048-VP2 (Figure 1D). Taken together, these results demonstrated the capability of

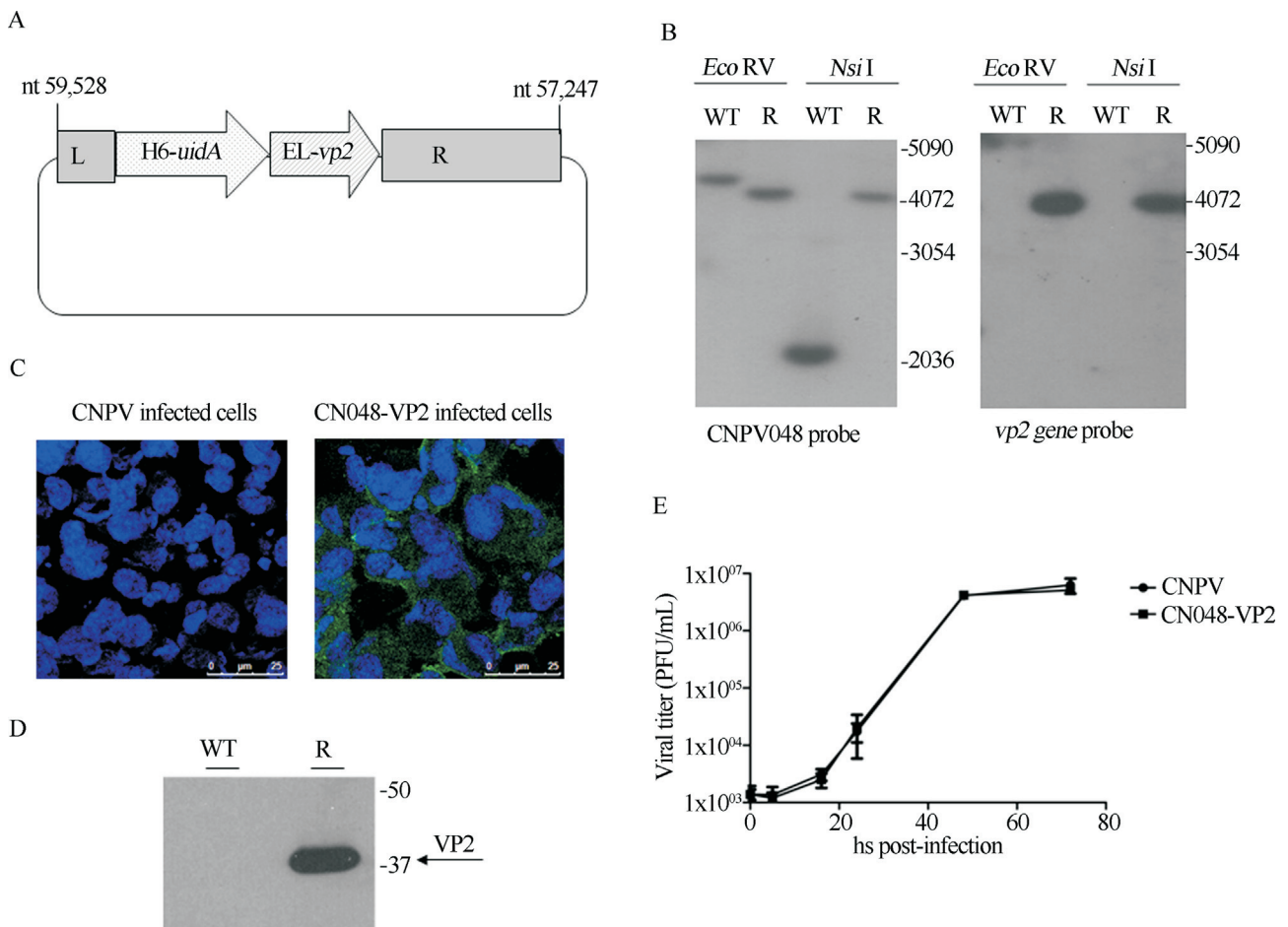


Figure 1 - Molecular characterization of CN048-VP2 recombinant virus. (A) Scheme of the transference vector TV-CN048-VP2. L: 600 bp fragment started in position 59,528. R: 1,700 bp fragment until position 57,247 encompassing the final region of CNPV048 gene, the complete CNPV047 gene and the initial region of CNPV046 gene. (B) Autoradiographies of Southern blot analysis using a 600 bp fragment of CNPV048 gene (positions 58,927 to 59,528) or *vp2* gene as ³²P-labelled probes. (C) Indirect immunofluorescence assay in DF1 cells infected with CN048-VP2 or non-recombinant CNPV viruses; white bars represents 25 μm. (D) Western blot assay: protein extracts were resolved in 12% SDS-PAGE and VP2 protein (indicated by an arrow) was detected using a rabbit polyclonal serum against IBDV-VP2 protein. Apparent molecular masses are indicated in kDa. WT: non-recombinant CNPV virus, R: recombinant CN048-VP2 virus. (E) Multiple-step growth curve of CNPV and CN048-VP2 in CEFs. The PFU/mL at each point is the mean of two independent experiments; errors bars represent standard deviations.

CN048-VP2 virus to *in vitro* express the IBDV VP2 protein with the predicted molecular weight for the mature protein. Thereafter, replication ability of recombinant and parental CNPVs was assessed by performing a multiple-step growth curve. Briefly, CEFs were infected at 0.01 plaque-forming units (PFU)/cell with CNPV or CN048-VP2 viruses. Supernatant and cells were collected together at different hours post infection (0, 5, 16, 24, 48 and 72 h) and frozen/thawed three times before titrating by duplicate in CEFs. The viral titer (PFU/mL) was calculated as the average value of the viral titers obtained in two independent experiments. Recombinant and wt CNPVs had the same growth curves indicating that the insertion of foreign sequences interrupting CNPV048 gene did not modify the viral replication capability in CEFs (Figure 1E). Then, the genetic stability of the recombinant viruses was evaluated by ten consecutive multiple-step growth passages on CEFs. After the 10th passage recombinant viruses produced 100% of blue plaques and the expression of VP2 protein was confirmed by WB (data not shown).

Then, the immunogenicity of CN048-VP2 was evaluated in SPF White Leghorn chickens randomly separated in four groups of five birds each. The chickens (11-days-old) were intramuscularly (i.m.) immunized with CNPV or CN048-VP2 (5.5×10^5 PFU/bird) or orally vaccinated with D78 live vaccine (2.7×10^3 PFU/bird) or PBS (50 μ L). All birds were boosted at 32 and 53 days-old with the same inocula. At different times (11, 32, 53 and 74 days old) blood samples were collected from the wing vein to evaluate the induced specific humoral response. The experiment was performed in the animal facilities at the Biotechnology Institute (INTA) in compliance with guidelines of Institutional Committee for the Use and Care of Experimentation Animals (CICUAE-INTA) that approved the protocol 18/2012 based on both National Institute of Health Guide for the care and use of laboratory animal and Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Sera from the same experimental group were pooled and analyzed by an *in vitro* IBDV neutralization test as described before (Jackwood *et al.*, 1982; Zanetti *et al.*, 2012a). As expected, neither the sera obtained from 11 days old birds (pre-immune status) nor the samples collected from CNPV or PBS groups showed IBDV-specific antibodies. However, IBDV neutralizing antibodies were detected in sera from chickens vaccinated with CN048-VP2 or D78. Besides, the seroneutralizing titer increased after each CN048-VP2 immunization indicating that the anti-vector immunity did not affect the efficacy of the booster vaccinations. The IBDV-D78 live vaccine induced higher level of neutralizing antibodies than the recombinant virus (Table 1).

At present, in the poultry industry, IBD is controlled by immunization with attenuated live or inactivated IBDV vaccines. However, these vaccines have several disadvantages. The inactivated vaccines are expensive and must be

Table 1 - IBDV-neutralizing titers in the sera of vaccinated birds

	Age (days)		
	32	53	74
CN048-VP2	16	32	512
IBDV-D78	512	1024	1024

Seroneutralizing titers are expressed as the reciprocal of the last serum dilution that completely neutralized 100 TCID₅₀ (50% tissue culture infective doses) units of IBDV strain LZD. CN048-VP2: recombinant CNPV virus expressing IBDV VP2 mature protein, IBDV-D78: live commercial IBDV vaccine (strain D78). IBDV-specific antibodies were negative both in the sera from 11 days old chicks (pre-immune) as in the samples collected from CNPV or PBS immunized birds.

administered to birds previously immunized with live vaccines (Müller *et al.*, 2003). Attenuated IBDV vaccines have to be applied once the IBDV maternal antibodies have decreased and could cause moderate bursal atrophy and/or immunosuppression. This fact allowed both the infection with other pathogens and poor immune response to other vaccines. More recently, the use of an immune complex vaccine (containing a live IBDV strain and an anti-IBDV hyperimmune serum, [Müller *et al.*, 2003]) or a turkey herpesvirus-based vector vaccine (recombinant HVT expressing IBDV-VP2 protein, [Poulet *et al.*, 2007]) has been implemented to the control of IBD in commercial poultry populations. Although these vaccines are effective in the presence of maternal antibodies inducing lifelong immunity, they have the major disadvantage of their high cost due to the addition of anti-serum (immune complex) or to the requirements of a cold chain to maintain the viability of HVT vector. In contrast, the high physical stability of CNPV-based viral vector allows the formulation of either freeze-dried or liquid vaccines with shelf-lives up to 2 years at room temperature (Poulet *et al.*, 2007).

In this report, we demonstrated that CN048-VP2 viruses were capable to elicit an IBDV specific humoral response with the presence of neutralizing antibodies in the vaccinated chickens. Due to neutralizing epitopes located on VP2 are discontinuous, it could be assumed that the VP2 protein expressed from CN048-VP2 had a correct conformational structure. In addition to that, we demonstrated that anti-canarypox vector immune response would not interfere with the following immunizations since the values of IBDV-seroneutralizing titers increased after each booster. This result agreed with observations of Poulet *et al.* (2007) who described that canarypox viral vectors are chosen for vaccination programs that require several boosters to achieve protective immunity. The immunization of chickens with CN048-VP2 viral vector elicited lower neutralizing antibody titers than replicative D78 live vaccine, but the poxvirus vector could also be inducing a cellular immune response contributing to protection against IBDV infections (Müller *et al.*, 2003). For that reason, further experiments have to be performed to evaluate both protection and cellular immune response induced by CN048-VP2

vaccination of chickens (with and without IBDV maternal antibodies). Besides, other routes of administration of CN048-VP2 consistent with the use in poultry industry such as subcutaneous immunization or wing-web scarification will be evaluated. Finally, a recombinant CNPV co-expressing cytokines with VP2 protein will be obtained with the aim to improve the specific immune response in chickens.

In conclusion, we demonstrated that recombinant canarypox viruses could be effective for developing novel vaccines against poultry diseases. To our knowledge this is the first report of immunization of chickens with canarypox virus-based vector.

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