

# Structure-dependent efficacy of infectious bursal disease virus (IBDV) recombinant vaccines

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## Abstract

The immunogenicity and protective capability of several baculovirus-expressed infectious bursal disease virus (IBDV)-derived assemblies as VP2 capsids, VPX tubules and polyprotein (PP)-derived mixed structures, were tested. Four-week-old chickens were immunised subcutaneously with one dose of each particulate antigen. VP2 icosahedral capsids induced the highest neutralising response, followed by PP-derived structures and then VPX tubules. All vaccinated animals were protected when challenged with a very virulent IBDV (vvIBDV) isolate, however the degree of protection is directly correlated with the levels of neutralising antibodies. VP2 capsids elicited stronger protective immunity than tubular structures and 3 µg of them were sufficient to confer a total protection comparable to that induced by an inactivated vaccine. Therefore, VP2 capsids represent a suitable candidate recombinant vaccine instead of virus-like particles (VLPs) for IBDV infections. Our results also provide clear evidence that the recombinant IBDV-derived antigens are structure-dependent in order to be efficient as vaccine components.

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## 1. Introduction

Infectious bursal disease (IBD) or Gumboro disease is a highly infectious virus disease of young chickens (3–6 weeks). It causes considerable economic losses to the poultry industry world-wide by causing a high rate of morbidity and mortality in an acute form or as a consequence of severe immunosuppression provoked by the destruction of immature B-lymphocytes within the bursa of Fabricius [1]. Infectious bursal disease virus-infected young birds (<2 weeks of age) generally show no clinical signs or mortality but may be immunodepressed during their lifetime which leads to an increased susceptibility to opportunistic infections and an ineffective response to vaccines. Control of IBD is primarily achieved by maternal-derived antibodies induced by live and inactivated vaccines given to breeder hens and by

vaccinating chicks with a live-attenuated strain of IBDV. Nevertheless, in recent years increasing problems have appeared due to the emergence of very virulent strains of IBDV (vvIBDV), which require increasingly effective vaccines to prevent disease since these strains were able to overcome maternally-derived antibodies [2,3]. However, vaccine strains aggressive enough to protect against these new viruses can themselves cause pathogenic effects. It is, therefore, important to develop new strategies of antigen presentation.

The etiological agent, infectious bursal disease virus (IBDV) is the prototype of the *Avibirnavirus* genus in the *Birnaviridae* family. Birnaviruses possess a bisegmented, double-stranded RNA (dsRNA) genome contained within a medium-sized (60–65 nm), unenveloped, icosahedral capsid but, unlike most dsRNA virus, single-shelled [4]. A 110-kDa polyprotein (PP) is autoproteolytically processed to yield VPX (also called pVP2) (≈48 kDa), VP3 (32 kDa) and VP4 (28 kDa) [5]. VPX is further cleaved by an unknown mechanism, rendering VP2 which, together with VP3, forms the capsid of the mature virion. The three-dimensional structure of IBDV has been determined [6,7]. The capsid has  $T = 13$  symmetry and is composed of 780 subunits in a regular

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array of 260 trimers. According to this accepted model, it is likely that those trimers, comprising VP2 and VP3, would form the Y-shaped trimers found at the inner surface.

VP2 is the main host protective antigen harbouring most of the neutralisation sites and has been the target protein for the development of subunit vaccines using a variety of different expression systems (for a review see [8]). Since the neutralising domains are highly conformational [9], successful vaccine development requires systems where the recombinant products mimic the authentic proteins and their conformation. In this context, virus-like particles (VLPs) have been powerful tools in a number of viral diseases [10–15] since they resemble the native viral capsids structurally and immunologically. For IBDV, the development of VLP-based vaccines would be of major interest because they give the correct immunogenic conformation that is crucial to promote a strong neutralising response and protection [8]. However, production of IBDV VLPs in the baculovirus system has proved to be quite inefficient and unproductive [16,17]. In fact, when we expressed the PP in insect cells, only a small amount of VLPs was obtained in addition to a diversity of particulate structures consisting of VLPs elongated in form of flexible tubular structures [18]. Recently, the production of a significant amount of baculovirus-expressed VLPs has been reported, although green fluorescent protein (GFP) had to be fused to the C terminus of VP3 for triggering a correct VLP assembly [19].

Expression of IBDV VPX gave rise to twisted tubular structures, 16–30 nm in diameter, without caps at their ends [7]. Expression of VP2 led to the formation of ring-shaped dodecahedral structures of about 23 nm in diameter, which were able to assemble into multi-capsids of 60–68 nm in diameter [7]. The 3D structure of small VP2 capsid, at 29 Å resolution revealed an icosahedral capsid, clustered as 20 protruding VP2 trimers, arranged in a  $T = 1$  lattice. The multi-capsid consisted of an icosahedron composed of 12 smaller  $T = 1$  dodecahedra, one in each vertex of the icosahedron leaving an internal cavity in the middle.

The aim of the present study was to assess and compare the vaccine potential of each of these recombinant constructions in chicken. Our findings confirm the relevance of the antigen conformation for IBDV vaccine development and indicate that VP2-based capsids elicit the best antibody response in terms of both quality and quantity.

## 2. Materials and methods

### 2.1. Animals

Four-week-old specific pathogen-free (SPF) White Leghorn light layers, hatched from SPF embryos from Lohmann GmbH (Cuxhaven, Germany), were used. The animals were hatched in isolation units and maintained in containment during experiments.

### 2.2. Cells, viruses and antibodies

*Spodoptera frugiperda* Sf9 cells were cultured in suspension at 27 °C using Grace's insect tissue culture medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL), 0.2% Pluronic F-68 and antibiotics. AcNPV and recombinant baculovirus were grown and assayed in Sf9 cells according to procedures described previously [20].

Vero cells were grown in Glasgow MEM (Sigma), supplemented with 10% FBS (HyClone). For neutralisation assays, 96 well plates (Nunc) were seeded and used 24 h later, at 100% confluency. The W2512 strain of IBDV adapted to grow in Vero cells, kindly provided by Fadly (ADOL, Michigan, USA) was used in the neutralisation assays. The vvIBDV strain VG-248, isolated in Girona (Spain), was used for challenge. It has been characterised as vvIBDV by RT-PCR RFLP and VP2 sequencing (GenBank accession number AY083925). The challenge virus was prepared by inoculating orally SPF birds at 4 weeks of age and harvesting the bursae at 3–4 days post-infection. Then, the bursae were homogenised in PBS (1:10 ratio), and 1 ml aliquots were stored at –80 °C.

Rabbit anti-VPX/VP2 monospecific serum was prepared as previously described by immunisation with selected peptides [21].

### 2.3. Expression and purification of IBDV-derived proteins in insect cells

The recombinant baculoviruses AcYM1-POLY, AcVPX-IBDV and AcVP2-IBDV, encoding Soroa-IBDV PP, VPX and VP2, respectively, were constructed as described previously [7,18]. For all protein expressions, 2-l fermentors containing Sf9 cells in mid-log growth in suspension cultures were infected at a multiplicity of infection (MOI) of 1 PFU per cell. Infected cells were harvested at 72 h post-infection and recombinant products were processed as described previously [7,18]. Briefly, lysates were subjected to centrifugation through a 25% (w/v) sucrose/PES buffer (25 mM PIPES, pH, 6.2, 150 mM NaCl, 20 mM CaCl<sub>2</sub>) cushion. The resuspended pellets were layered onto a 25–50% (w/v) sucrose/PES gradient. Fractions were analysed by SDS/PAGE in 11% gels. Proteins were either stained with Coomassie blue or analysed on an immunoblot with anti-VPX/VP2 rabbit serum [18]. Immunoreactive fractions of each construction were pooled, diluted in PES and concentrated by centrifugation at 125,000 × *g* for 3 h at 4 °C. The pellets were resuspended in PES at 0.5 mg/ml. Purified samples were adsorbed to carbon-coated grids, stained with 2% (w/v) uranyl acetate, and examined at 40,000× magnification in a JEOL 1200 EXII electron microscope. The presence of VP2-derived multi-capsids was also confirmed by electron cryomicroscopy as described previously [7]. The total protein concentration was determined by the Bio-Rad protein assay using bovine serum albumin as the standard.

#### 2.4. Immunization procedure

The required amount of antigen was emulsified with oil (W/O vaccines) in 0.5 ml for each vaccinal dose. A commercial inactivated W/O vaccine, HipraGumboro-BPL2 (Laboratorios Hipra S.A., Girona, Spain), which contained whole inactivated IBDV virus, was used as a standard vaccine.

First, 4-week-old SPF chicks were divided into six groups. Three groups of 10 birds each were inoculated with the corresponding recombinant vaccine containing 25 µg of VP2 (group A) or VPX (group B) or PP (group C). Commercial vaccine BPL2 was administered to another group of 10 animals (group D). A group of 13 birds was non-vaccinated and challenged as a challenge control (group E) and a group of 6 birds was kept as a non-vaccinated non-challenged control (group F).

Second, the dose/response was also tested. Groups of 10 birds were inoculated with VP2 (A) and PP (B) experimental vaccines at different doses: 9 µg (group A.1 and B.1), 3 µg (group A.2 and B.2) and 1 µg (group A.3 and B.3). Other groups of 10 birds were used for the commercial vaccine (group C) and as a challenge control (group D) and another group of 4 animals was kept as a non-vaccinated non-challenged group (group E).

The potency of the experimental vaccines was checked according to the European Pharmacopoeia method for batch testing of IBDV inactivated vaccines [22]. Four-week-old SPF birds were inoculated by subcutaneous route in the back of the neck with one dose of the vaccine to be tested. At 4 weeks post-vaccination, the animals were blood sampled from the wing vein and sera from birds of the same group were pooled and assayed by serum neutralisation (SN).

#### 2.5. Challenge infection

As an additional measure of vaccine efficacy, not prescribed in the European Pharmacopoeia, a challenge test was performed. At 4 weeks post-vaccination and after blood sampling, 8-week-old birds were inoculated by oral route with 0.2 ml of a 1/20 dilution in PBS of a bursal homogenate of vvIBDV strain VG-248. The typical IBD symptoms were recorded: ruffled feathers, depression, head between the shoulders and death. At 10 days post-challenge, the surviving animals were sacrificed and necropsied. The bursa of Fabricius (BF)/body weight (BW) ratio was calculated using the formula: (BF weight (in g)/BW (in g)) × 1000. A BF/BW ratio below 2 was considered as a marker of bursal atrophy, which meant that the challenge virus had reached the bursa. In the second trial, a portion of the bursa was kept frozen at -80 °C, and tested later for the presence of IBDV by RT-PCR.

#### 2.6. ELISA

Antibodies against IBDV host-protective antigen VP2 were detected by an indirect enzyme-linked immunosor-

bent assay (ELISA) that used recombinant VPX as antigen source as previously described [23]. Titres were expressed as -log of the serum dilution that yielded absorption values three times above the blank (pre-immunisation serum).

#### 2.7. Virus-neutralisation assay

In a 96-well plate, two-fold dilutions of the pooled sera obtained from every group of vaccinated animals were added to a 100 TCID<sub>50</sub> units of IBDV strain W2512. The mixture of serum dilution and virus was incubated at 37 °C for 1 h, and then the content of the wells was added to confluent Vero cells. Cells were incubated at 37 °C until cytopathic effect was observed (usually 7 days). The SN titre was the highest serum dilution that prevented the appearance of cytopathic effect. A standard positive control serum, containing by definition 10,000 Ph. Eur. units, was included [24]. A vaccine complies with the test if the pooled serum from the vaccinated chickens is at least 10,000 Ph. Eur. units/ml. The SN titre in Ph. Eur. units/ml is given by  $X/S \times 10,000/V$ , where  $X$  is the reciprocal end-point dilution of the unknown serum,  $S$  the reciprocal end-point dilution of the standard serum and  $V$  the volume to which the standard serum is reconstituted in millilitres.

#### 2.8. RT-PCR assay

IBDV VP2-specific primers were chosen from the VP2 gene, the upstream primer was IBDV-804 (5'-GTAACAAT-CACACTGTTCTCAG-3') and the downstream primer was IBDV-1055 (5'-GATGGATGTGATTGGCTGGG-3'). Bursae were harvested at 10 days post-challenge from survivors of the IBDV challenge. About 250 mg of tissue were homogenised in 200 µl of PBS and suspensions were clarified by centrifugation. Viral RNA was extracted from each supernatant with Trizol LS reagent (Invitrogen) and precipitated with isopropanol at -20 °C. The resulting RNA pellet was washed once with 70% ethanol and dissolved in 25 µl of nuclease-free water. Uninfected bursa controls were always processed concomitantly.

RT-PCR reactions were performed using SuperScript<sup>TM</sup> one-step RT-PCR (Invitrogen) and 2.5 µl of RNA in a 50 µl reaction volume. The reaction mix contained 0.2 µM each of the primers. Reverse transcription was carried out at 50 °C for 20 min and the resulting cDNA amplified with the following temperature profile: 94 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 1 min 30 s, and elongation at 72 °C for 1 min and ending with a final elongation for 5 min.

### 3. Results

#### 3.1. Preparation of IBDV-derived antigens used for inoculation of chickens

Infections of Sf9 cells with the corresponding recombinant baculoviruses resulted in the expression of VP2, VPX



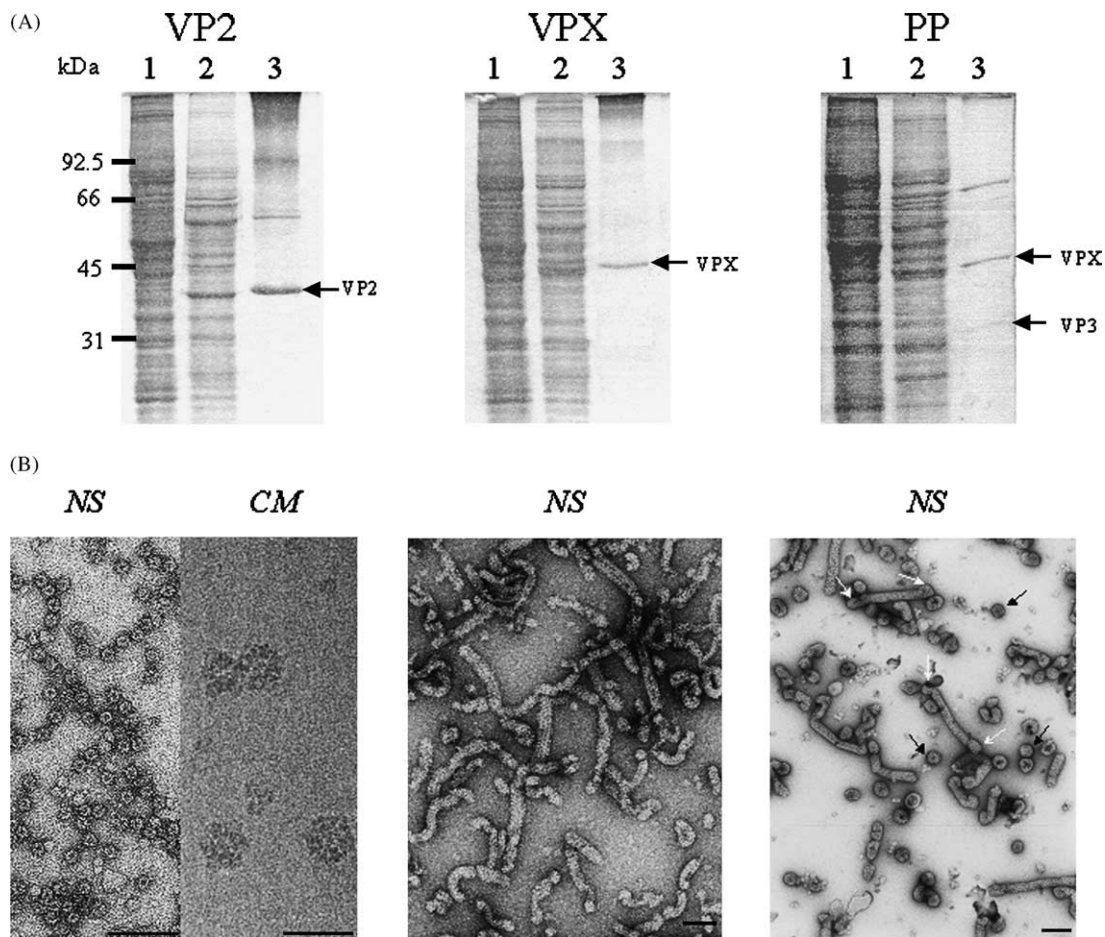


Fig. 1. Expression in insect cells, purification and structural analysis of IBDV VP2, VPX and PP. (A) Coomassie-stained 11% SDS-PAGE gels showing the expression and purification by sucrose-gradient centrifugation (see Section 2) of IBDV VP2 (left panel), VPX (middle panel) and PP (right panel). Lane 1: extracts from non-infected Sf9 cells as negative controls; lane 2: extracts from Sf9 cells infected with the corresponding recombinant baculovirus (AcVP2-IBDV, AcVPX-IBDV or AcPOLY-IBDV expressing VP2, VPX or PP, respectively); and lane 3: purified proteins. Arrows indicate VPX, VP2 and VP3 proteins. Molecular masses of marker proteins are indicated in kDa at the left of the first panel. (B) Electron microscopy of purified recombinant IBDV-related structures. Left panel corresponds to VP2 assemblies analysed by negative staining (NS) and cryoelectron microscopy (CM). Middle panel shows VPX-derived tubules. PP-derived structures are presented in the right panel. VLPs are indicated by black arrows and white arrows point out to caps at the end of the tubules; bars, 100 nm.

and PP particles. After purification, the yields for every particle were similar, approximately 4 mg of each antigen/l of culture. The purity and protein composition of all recombinant structures used in this study were proved by electrophoretic analysis (Fig. 1A) and Western blot (data not shown). Electron microscopy observations confirmed that expression of PP gave rise to VLPs and capped tubules, expression of VPX rendered flexible tubules and expression of VP2 resulted in the production of 23 nm capsids and 65 nm multi-capsids, detectable only by cryoelectron microscopy (Fig. 1B).

### 3.2. Immune response of chickens vaccinated with recombinant IBDV-derived structures

SPF chickens were immunised once subcutaneously with 25  $\mu$ g of each recombinant structure. Another group was

inoculated with commercial inactivated IBDV vaccine and two groups of negative controls remained unvaccinated, one as sentinel. Antibody responses were monitored in serum samples taken 4 weeks after immunisation by measuring the anti-VP2/VPX specific antibodies using a VPX-based ELISA and by testing the presence of neutralising antibodies. As shown in Fig. 2, all immunised animals developed high levels of specific antibodies to VP2/VPX with titres about 4 log<sub>10</sub>, which did not differ significantly among groups vaccinated with recombinant antigens (VP2, VPX or PP) or inactivated viruses (commercial vaccine). In contrast, some differences could be observed among the different vaccine groups with regard to the development of neutralising antibodies. VP2-based vaccine produced significantly greater neutralising antibody titres (5.31 log<sub>10</sub> or 160,000 Ph. Eur. units/ml) than VPX, PP or even the inactivated vaccine, with neutralising titres of 4.1 log<sub>10</sub> (10,000

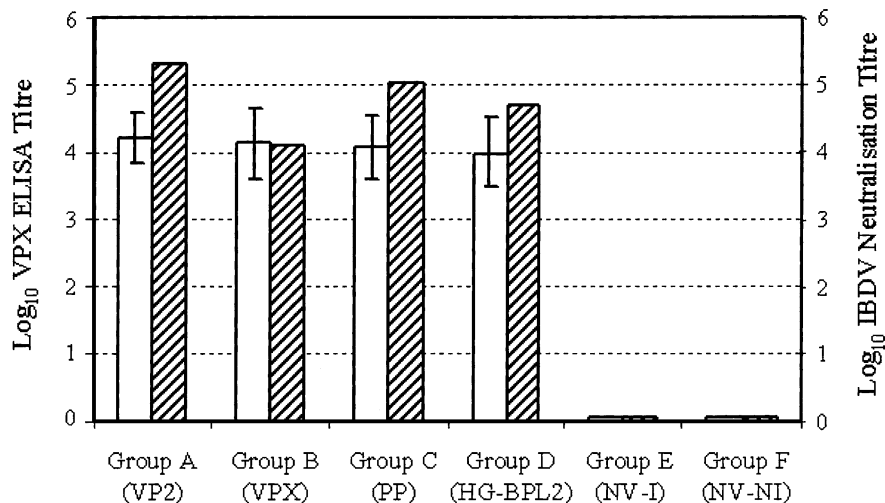


Fig. 2. Comparison of serum VPX-specific (open bars) and neutralising (striped bars) antibody responses after 4 weeks post-immunisation in chickens vaccinated with VP2 capsids (group A), VPX tubules (group B), PP-derived structures (group C) or commercial inactivated vaccine HG-BPL2 (group D). Control groups E and F were non-vaccinated and infected (NV-I) and neither vaccinated nor infected (NV-NI), respectively. Anti-VPX titres for each group are represented as the geometric mean  $\pm$  S.D. on a  $\log_{10}$  scale. Neutralising titres are expressed as the  $-\log_{10}$  of the highest dilution which prevented the appearance of cytopathic effect for pooled serum samples of each group.

Ph. Eur. units/ml),  $5.01 \log_{10}$  (80,000 Ph. Eur. units/ml) and  $4.7 \log_{10}$  (40,000 Ph. Eur. units/ml), respectively. In spite of these differences, all the recombinant vaccines meet the potency requirements stated by the European Pharmacopoeia (SN titres  $\geq 10,000$  Ph. Eur. units/ml).

### 3.3. Protection efficacy against vvIBDV challenge

Chickens were challenged with a vvIBDV isolate (strain VG-248) at the age of 8 weeks. Protection was measured by monitoring clinical manifestations and bursal damage at 10 days post-infection (Table 1). Recombinant vaccines conferred protection to all the vaccinated chickens, as did the inactivated commercial vaccine. In contrast, all animals in the non-vaccinated and infected group succumbed to the infection, showing mortality and morbidity rates of 46 and 100%, respectively. Only one bird from the VP2-vaccinated group showed detectable bursal atrophy (BF/BW ratio  $< 2$ ), identical score to that obtained with the inactivated vaccine (1 out of 10 animals). Two PP-vaccinated birds had bur-

sal atrophy and a slight decrease of the mean BF/BW ratio ( $3.67 \pm 1.53$ ) in comparison with the uninfected group F ( $4.11 \pm 1.30$ ). The lowest protection was obtained in the VPX-vaccinated group where 30% of the animals showed bursal atrophy and the mean BF/BW ratio decreased until  $2.61 \pm 1.00$ . All the surviving chickens (7/7) from the non-vaccinated group E showed severe gross bursal lesions and a drastic reduction in their BF/BW ratio ( $0.96 \pm 0.15$ ) indicating massive IBDV infection. Representative bursae and spleens from every chicken group are shown in Fig. 3. In conclusion, the best recombinant immunogen in terms of protection against mortality and prevention of bursal damage was the VP2-derived capsids, followed by PP-derived structures.

### 3.4. Dose-effect of VP2- and PP-derived structure on the humoral responses

Then, a dose-effect was determined for the VP2 capsids and PP-derived structures. Three doses of 9, 3 and 1  $\mu\text{g}$ , were

Table 1  
Protection of SPF chickens vaccinated with recombinant vaccines or inactivated vaccine

Group (antigen)	Mortality (%)	No. of animals with severe clinical signs <sup>a</sup>	BF/BW ratio <sup>b</sup>	No. of animals with BF/BW ratio $< 2$ <sup>c</sup>
A (VP2)	0/10 (0)	0/10	$3.91 \pm 1.53$	1/10
B (VPX)	0/10 (0)	0/10	$2.61 \pm 1.00$	3/10
C (PP)	0/10 (0)	0/10	$3.67 \pm 1.53$	2/10
D (HG-BPL2)	0/10 (0)	0/10	$4.36 \pm 1.42$	1/10
E (NV-I)	6/13 (46)	7/7	$0.96 \pm 0.15$	7/7
F (NV-NI)	0/6 (0)	0/6	$4.11 \pm 1.30$	0/6

<sup>a</sup> Number of surviving birds exhibiting severe clinical signs/number of surviving birds.

<sup>b</sup> Average of BF/BW ratio (bursal weight/body weight  $\times 1000$ )  $\pm$  standard deviation of the surviving birds.

<sup>c</sup> Number of animals with BF/BW ratio lower than 2/number of surviving birds. BF/BW ratio lower than 2 indicates bursal atrophy.

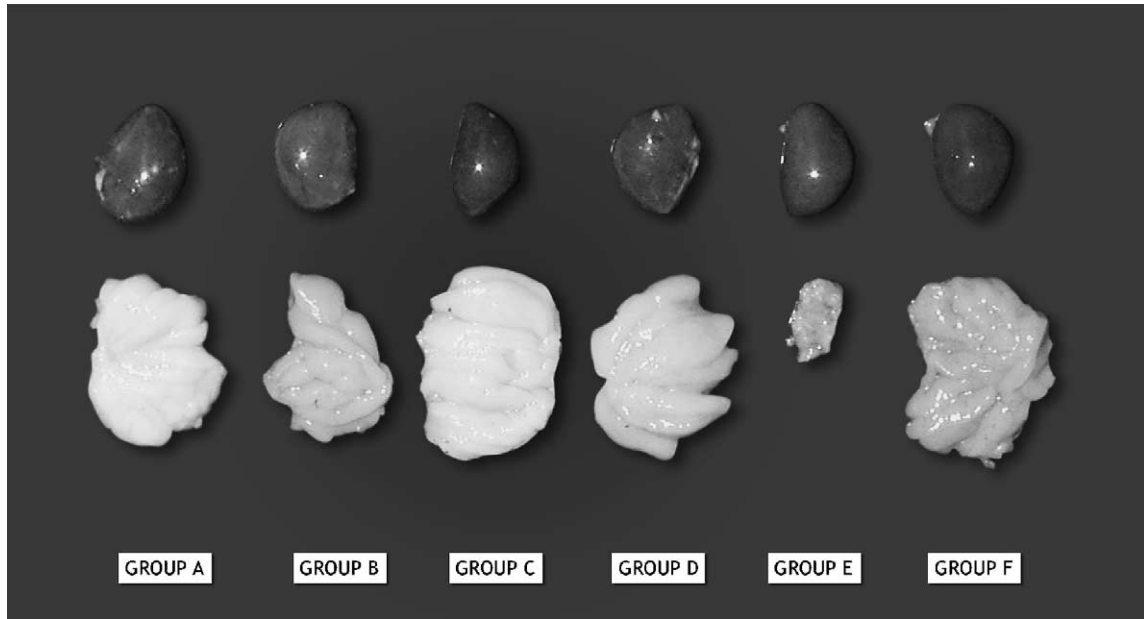


Fig. 3. Representative bursae and spleens from chickens vaccinated with 25  $\mu\text{g}$  of VP2 capsids (group A), VPX tubules (group B), PP-derived structures (group C) or with commercial inactivated vaccine (group D), and non-vaccinated (group E) 10 days after challenge with vvIBDV. Group F: normal control (non-vaccinated and non-infected). Spleens (upper positions) were included for size comparisons.

administered to chickens in a single subcutaneous dose. The antibody levels were measured by ELISA and in vitro neutralisation at week 4 (Fig. 4). As before, chicken vaccinated with VP2 capsids showed higher responses than those vaccinated with PP-derived structures at equal dose. Unexpectedly, birds immunised with 9  $\mu\text{g}$  of PP responded to a lower extent than the other groups. The neutralisation titres were quite similar in all the vaccinated groups (pooled serum titres of  $4.69 \log_{10}$ , 40,000 Ph. Eur. units/ml) except in group A.1 (9  $\mu\text{g}$  of VP2), which responded with slightly higher neutralising titres ( $5.3 \log_{10}$  or 160,000 Ph. Eur. units/ml) comparable to the commercial killed vaccine ( $5.6 \log_{10}$  or 320,000 Ph. Eur. units/ml). Non-vaccinated animals (groups D and

E) did not show specific antibodies or neutralising antibodies against IBDV. Therefore, no clear dose-effect was observed suggesting that the amount of VP2 capsids could be reduced from 25 to 9  $\mu\text{g}$  without changes in the neutralising titres and that even a dose of 1  $\mu\text{g}$  of recombinant antigens could be immunogenic.

### 3.5. Protection efficacy at different VP2- and PP-derived structure doses

As before, 8-week-old chickens were challenged with vvIBDV except group E that remained as a sentinel group. After challenge, all the animals immunised with the

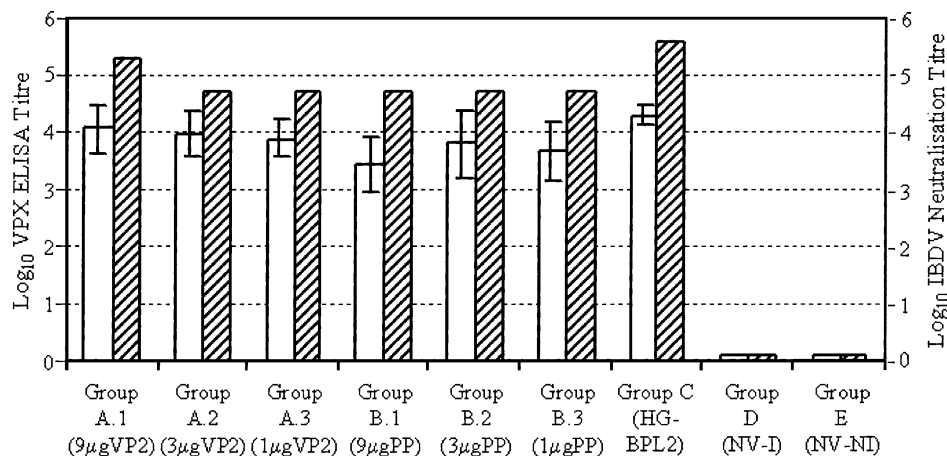


Fig. 4. Serum VPX-specific (open bars) and neutralising (striped bars) antibody responses in a dose-related experiment with chickens vaccinated with 9, 3 and 1  $\mu\text{g}$  of VP2 (groups A.1, A.2 and A.3, respectively) or PP (groups B.1, B.2 and B.3, respectively). HG-BPL2 group D was included as a positive control and NV-I group E and NV-NI group F as negative controls.

Table 2  
Protective capabilities of VP2 capsids and PP structures in a dose-related experiment

Group (dose-antigen)	Mortality (%)	No. of animals with severe clinical signs <sup>a</sup>	BF/BW ratio <sup>b</sup>	No. of animals with BF/BW ratio <2 <sup>c</sup>	IBDV in bursa <sup>d</sup>
A.1 (9 µg VP2)	0/10 (0)	0/10	4.66 ± 1.43	0/10	0/10
A.2 (3 µg VP2)	0/10 (0)	0/10	4.84 ± 1.34	0/10	1/10
A.3 (1 µg VP2)	0/10 (0)	1/10	4.40 ± 1.76	1/10	4/10
B.1 (9 µg PP)	0/8 (0)	1/8	4.71 ± 1.40	0/8	8/8
B.2 (3 µg PP)	0/10 (0)	2/10	3.86 ± 1.52	1/10	10/10
B.3 (1 µg PP)	0/10 (0)	5/10	4.00 ± 1.37	1/10	10/10
C (HG-BPL2)	0/10 (0)	2/10	4.89 ± 0.94	0/10	1/10
D (NV-I)	9/10 (90)	1/1	1.06	1/1	1/1
E (NV-NI)	0/4 (0)	0/4	4.23 ± 0.54	0/4	0/4

<sup>a</sup> Number of surviving birds exhibiting severe clinical signs/number of surviving birds.

<sup>b</sup> Average of BF/BW ratio (bursal weight/body weight) × 1000 ± standard deviation of the surviving birds.

<sup>c</sup> Number of animals with BF/BW ratio lower than 2/number of surviving birds. BF/BW ratio lower than 2 indicates bursal atrophy.

<sup>d</sup> Detected by PCR (see Section 2). Number of animals positive for IBDV in bursa tissue vs. number of surviving animals at 10 days post-challenge.

recombinant or commercial vaccines survived. Mortality and morbidity rates due to IBDV in the non-vaccinated and infected group D were 90 and 100%, respectively. Clinical symptoms were totally prevented in chickens immunised with every dose of VP2 capsid, except for one animal in the group vaccinated with the lowest dose (1 µg). PP-immunised groups were not so well protected, since severe IBD symptoms were observed in some animals (Table 2). Two out of 10 birds vaccinated with the commercial vaccine showed IBD symptoms after infection showing that the challenge conditions were extremely severe.

The BF/BW ratios of chickens vaccinated with commercial and recombinant vaccines at all doses were similar (from 3.86 ± 1.52 to 4.89 ± 0.94) to those of control group E (3.23 ± 0.54) and significantly higher ( $P < 0.05$ ) than that of challenge control group D (1.06). The highest score was achieved in chickens vaccinated with 3 µg of VP2 capsid (4.84 ± 1.34), which was similar to that of the commercial vaccine group (4.89 ± 0.94). Only three animals from groups vaccinated with 1 µg of VP2 or 3 and 1 µg of PP showed indexes lower than 2 indicating bursal atrophy.

An RT-PCR analysis was also performed on the bursae on each surviving bird to study the presence of IBDV (Table 2). No IBDV was detected by RT-PCR in the bursae of chickens vaccinated with 9 µg of VP2 capsid. Ten percent of animals vaccinated with 3 µg of VP2 or with the commercial vaccine and 40% of birds vaccinated with 1 µg of VP2 showed virus in their bursae, indicating a significant VP2 dose–effect. In contrast, all the PP-vaccinated chickens showed presence of IBDV in bursa, independently of the dose.

#### 4. Discussion

The immunogenicity and protective efficacy of different IBDV-derived particles: VP2 capsids, VPX tubules and PP-derived structures were assessed in chickens. To our knowledge, this is the first report that compared the immunogenicity of different assemblies derived from equiva-

lent sequences. The results established that all the antigens tested were effective at inducing humoral responses. However, the virus-neutralising capacity of the antibodies induced by VP2 icosahedral capsids was significantly stronger compared with that induced by VPX tubules or PP-derived structures at the same doses. This superior immunogenicity of VP2 (456 aa) was observed despite sharing the same sequence except for being 60 amino acids shorter at the C terminus (VPX: 516 aa). It has been demonstrated that the major neutralising sites are conformation-dependent and an incorrect VP2 folding causes a lack of immunogenicity [9,25], therefore this result indicates that neutralising epitopes are not as well represented on tubules as they are on icosahedral particles.

According to the 3D structures of virions [6,7] and VP2 capsids [7], a similar topography between them is observed, as both structures display protruding trimeric units equivalent in size and morphology. Therefore, since VP2 seems to retain its native structure in these recombinant capsids, one can expect them to elicit antibodies against conformational epitopes identical to those triggered by complete virions. Although precise definition and location of the sequences forming neutralising domains must wait for the molecular structure of IBDV, it seems reasonable to speculate that the major neutralisation and antigenic variation site (hypervariable region), mapped between amino acids 206 and 350 [26,27], would be forming part of the protruding regions of each trimeric unit. A two-dimensional analysis of VPX- or PP-derived tubules showed that the conformation of the trimeric capsomers are quasi-equivalent but not identical in tubules and capsids [7,18]. In VPX tubules, trimers are arranged forming hexamers, whereas in VP2 capsids, trimers are ordered in form of pentamers, indicating that interactions between adjacent trimers should be different in both structures. Those differences in conformation and orientation seem to have a significant effect on the specificity of the antibodies elicited and the magnitude of the immune response against conformation-dependent epitopes of IBDV.



PP-derived structures were less effective than VP2 capsids at eliciting a neutralising response but clearly superior to VPX tubules, probably because PP particles still contain a percentage of VLPs. PP expressed in other systems as fowlpox virus [28] and Semliki Forest virus [29] failed to induce IBDV neutralising antibodies in immunised chickens. These results may reflect differences in the processing and assembly of the corresponding recombinant proteins or the absence of VLPs. PP-derived VP2 produced in baculovirus would be more correctly folded or in higher amounts than in the other vector virus systems.

The choice of 8-week-old birds for challenge was forced by the timing imposed by the European Pharmacopeia regulations for vaccine potency assay. Still, chickens were efficiently challenged, as demonstrated by the clear clinical symptoms and mortality caused by the virus in the control groups. The protection given by low doses of PP-derived vaccine was not as potent as that induced by VP2 capsids, showing only partial clinical protection and bursal atrophy. Those differences in protection observed between VP2 and PP antigens despite to elicit similar neutralising response, could be due to the different conformation adopted by the neutralising epitopes or to other immune mechanisms that could be triggered by VP2 capsids. In fact, although cell-mediated immunity to IBDV has not been well characterised yet, there are some evidences that suggest the contribution of more than one immune effector mechanism in protection. Recombinant fowlpox virus [30,31] and Marek's disease virus [32] expressing IBDV VP2, as well as DNA vaccines [33], were able to induce protection with low levels or in the absence of detectable antibodies indicating that antibody-independent protection mechanisms are also involved. From our data, it seems reasonable to conclude that icosahedral structures as VLPs and VP2 capsids are more effective than tubular assemblies at inducing an IBDV protective response.

Recombinant vaccines must have selective advantages over traditional vaccines to be accepted by the poultry industry. Together with considerations as safety and efficacy, the cost-effectiveness and easiness of production are critical factors to make a commercially feasible vaccine. A 4 mg yield of highly-purified VP2 capsid per litre of culture, together with a low dose (between 3 and 9 µg per animal) make these antigens promising candidates to develop a cheap and attractive vaccine. However, the purification process based on sucrose-gradient centrifugation is tedious, difficult to scale and comparatively expensive. To circumvent this, additional studies are required to analyse the protective abilities of non-purified VP2 capsids, as successful vaccination using this approach has been reported for other viral diseases [34,35].

Ease of vaccine administration is also an important requisite for considering it feasible for poultry industry. Subcutaneous injection is laborious and time-consuming because large populations of chicken must be vaccinated at the same time. So, other post-hatch delivery system such as spray,

drinking water or eye drop, should be explored to distribute recombinant VP2 capsids simultaneously to large numbers of birds.

In any case, our results demonstrate that the experimental vaccines, specially the VP2-derived one, meet the potency requirements of the European Pharmacopeia for registration. In the future, they will be tested in breeders, which is the main target for inactivated vaccines. We can anticipate that VP2 capsids will elicit neutralising antibodies which should transfer to progeny by analogy with previous reports, in which maternal antibodies from baculovirus-expressed PP and VP2 were effectively passed on to offsprings [36,37].

Finally, this study clearly reveals that a proper icosahedral conformation of the immunogens is essential for the stimulation of an efficient protective immune response against IBDV, whereas tubular structures are not so effective. VP2 capsids could provide a safe and efficacious alternative to inactivated IBDV vaccines and could replace a VLP-based candidate vaccine. An extra advantage would be that a VP2 capsid vaccine could be administered to avian population without compromising disease surveillance by serological screening. This would be very useful for the diagnosis and the control of IBDV outbreaks because it would permit discrimination between naturally infected and vaccinated animals by the detection of antibodies against other viral proteins, for instance, VP3.

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