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Comparison of homologous and heterologous prime-boost immunizations combining MVA-vectored and plant-derived VP2 as a strategy against IBDV

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ABSTRACT

Different immunogens such as subunit, DNA or live viral-vectored vaccines against Infectious Bursal Disease virus (IBDV) have been evaluated in the last years. However, the heterologous prime-boost approach using recombinant modified vaccinia Ankara virus (rMVA), which has shown promising results in both mammals and chickens, has not been tried against this pathogen yet. IBD is a highly contagious and immunosuppressive disease of poultry that affects mainly young chicks. It is caused by IBDV, a double-stranded RNA virus carrying its main antigenic epitopes on the capsid protein VP2. Our objective was to evaluate the immune response elicited by two heterologous prime-boost schemes combining an rMVA carrying the VP2 mature gene (rVP2) and a recombinant VP2 protein produced in *Nicotiana benthamiana* (pVP2), and to compare them with the performance of the homologous pVP2-pVP2 scheme usually used in our laboratory. The SPF chickens immunized with the three evaluated schemes elicited significantly higher anti-VP2 antibody titers ($p < 0.001$) and seroneutralizing titers ($p < 0.05$) and had less T-cell infiltration ($p < 0.001$), histological damage ($p < 0.001$) and IBDV particles ($p < 0.001$) in their bursae of Fabricius when compared with control groups. No significant differences were found between both heterologous schemes and the homologous one. However, the rVP2-pVP2 scheme showed significantly higher anti-VP2 antibody titers than pVP2-rVP2 and a similar tendency was found in the seroneutralization assay. Conversely, pVP2-rVP2 had the best performance when evaluated through bursal parameters despite having a less potent humoral immune response. These findings suggest that the order in which rVP2 and pVP2 are combined can influence the immune response obtained. Besides, the lack of a strong humoral immune response did not lessen the ability to protect from IBDV challenge. Therefore, further research is needed to evaluate the mechanisms by which these immunogens are working in order to define the combination that performs better against IBDV.

1. Introduction

Infectious Bursal Disease (IBD) is a highly contagious and immunosuppressive disease of poultry that affects mainly young chicks [1]. It is caused by the Infectious Bursal Disease Virus (IBDV), a bi-segmented double-stranded RNA virus [2] carrying its main antigenic epitopes on the capsid protein VP2 [3]. This viral pathogen targets IgM bearing B-lymphocytes in the bursa of Fabricius (BF) [4]. The economic losses caused by IBDV are due to increased susceptibility to other pathogens and poor vaccine efficacy because of the immunosuppression acquired by the animals, but can also be caused by high mortality in flocks infected with very virulent IBDV strains [5].

IBD is essentially controlled through vaccination. Vaccines against IBDV should be given relatively early because of the chicken's susceptibility period to the disease. At present, the most widely used immunogens are based on live attenuated IBDV

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strains, even though they may cause immunosuppression and bursal atrophy, and are effective only when IBDV maternal antibodies have decreased [6].

In order to overcome these issues, different immunogens such as subunit, DNA or live viral-vectored vaccines have been rationally designed and evaluated [6,7]. Our group has had successful experiences immunizing chickens with VP2 protein produced both in *Nicotiana benthamiana* plants [8] and through non replicative poxviral vectors [9–11].

To induce broader immune responses, different heterologous prime-boost regimes have been successfully used in mammals [12]. Particularly, recombinant modified vaccinia Ankara virus (rMVA) has shown to be more effective when used as a heterologous boost after priming with the same antigen in a different form [13], both in mammal [14,15] and avian [16] models. On the other hand, a poxvirus prime-protein boost scheme has been considered the most successful vaccination protocol against HIV-1 [17].

Thus, having a consistently immunogenic protein as VP2 from *N. benthamiana* (pVP2), our objective was to investigate whether its combination with a recombinant MVA expressing VP2 (rVP2) in a heterologous prime-boost scheme could elicit a stronger immune response than the one induced by the homologous scheme based on pVP2. This is, to the best of our knowledge, the first trial combining a recombinant MVA with a recombinant protein as a strategy against IBDV in chickens.

2. Materials and methods

2.1. Animals

Specific pathogen free (SPF) embryonated eggs were purchased from Instituto Rosenbusch S.A. (Argentina) and hatched in an automatic incubator (Yonar, Argentina). Chickens were housed in individual cages with food and water provision *ad libitum*. All procedures involving the use of animals were performed in agreement with institutional guidelines and approved by the Institutional Committee for the Care and Use of Experimental Animals (CICUAE – CICVyA – INTA).

2.2. Immunogens

Recombinant MVA viruses have been previously obtained in our laboratory: rVP2 [9] and MVA-GFP (rGFP) carry the coding sequence of IBDV-VP2 mature protein or the gene coding for the green fluorescent protein (GFP), respectively.

MVA viruses were amplified in primary Chicken Embryo Fibroblasts (CEFs) cultured in Dulbecco's Modified Eagle Medium (D-MEM) (Thermo Fisher Scientific, USA) supplemented with 2% fetal bovine serum (Internegocios, Argentina) at 37 °C with 5% of CO_2. When 90–100% cytopathic effect (CPE) was detected, cells and supernatant were collected together and 3 freeze-thaw cycles were performed to release the virus.

The other antigen, pVP2, which also had its GFP-control (pGFP), was extracted from pEAQ vector-infiltrated leaves [18] in a blender with 3 volumes of chilled buffer containing phosphate buffered saline (PBS) pH 7.3, complete EDTA-free protease inhibition cocktail tablets (Roche, Germany) and 0.04% antifoam O-30 (Sigma Aldrich, USA), and the expression of VP2 was evaluated through Western Blot as described in [8].

2.3. Experimental design

Thirty chicks were randomly divided in 5 groups and immunized intramuscularly (IM) as stated in Table 1. Chickens were bled periodically by the wing vein, and the sera obtained were stored at −20 °C until use.

Table 1
Experimental design.

Group	1st inoculation	2nd inoculation	Challenge
1	pVP2 (8 μg)	pVP2 (8 μg)	
2	rVP2 (6.5×10^5 PFU)	pVP2 (8 μg)	
3	pVP2 (8 μg)	rVP2 (6.5×10^5 PFU)	8.25×10^6 PFU/ chicken
4	rGFP (6.5×10^5 PFU)	rGFP (6.5×10^5 PFU)	
5	pGFP (8 μg)	pGFP (8 μg)	

Six specific pathogen free chickens were used in each group. Inoculations were performed at 14 days post hatch (1st) and 28 days post hatch (2nd). Animals were challenged three weeks after the 2nd inoculation and euthanized 5 days post challenge.

Three weeks after the boost immunization animals were challenged orally with 8.25×10^6 PFU of Winterfield strain of IBDV and euthanized five days post challenge.

2.4. Anti-IBDV and anti-VP2 antibodies detection

Sera obtained before challenge were analyzed using the IDEXX IBD test kit (IDEXX Laboratories, USA) following manufacturer's instructions.

In addition, sera were tested for specific anti-VP2 antibodies using an indirect ELISA based on subviral particles (SVP) formed by IBDV VP2. Briefly, 96-well Maxisorp™ Nunc™ flat-bottom plates (Thermo Scientific, USA) were coated with 25 ng of SVPs *per* well in 0.1 M carbonate-bicarbonate buffer, pH 9.6, overnight at 4 °C. After blocking with 4% skim milk in PBS–T (0.05% Tween 20), plates were subsequently incubated with 3-fold serial dilutions of sample sera, and 1/3000 dilutions of goat anti-chicken IgG antibodies coupled to horseradish peroxidase (Bethyl Laboratories, USA). Revealing step was performed using ABTS substrate (Sigma-Aldrich, USA)-H_2O_2 in citric acid buffer, pH 5. Reading was done at 405 nm after 20 min of incubation. Antibody titers were calculated as the inverse of the highest dilution with an OD_{405nm} value above the mean +3.5 SD of the negative control groups.

2.5. Seroneutralization assay

Sera were inactivated at 56 °C for 30 min and twofold serially diluted from 1/4 to 1/8192 in D-MEM. Dilutions were incubated with 100 $TCID_{50}$ of Winterfield strain of IBDV for 1 h at 37 °C in 96-well plates (Greiner bio-one, Germany). After that, 100 μl of a cell suspension of 1.5×10^6 CEFs/ml were added to each well. The cell suspension was prepared in D-MEM supplemented with 4% fetal bovine serum (FBS), 25 mM HEPES and a mixture of antibiotic/antimycotic (Gibco®, USA). Cells were cultured at 37 °C with 5% CO_2 for 4 days, when CPE was observed. Seroneutralizing antibody (SN) titers were calculated as the inverse of the highest dilution showing no CPE.

2.6. Lymphocyte isolation and flow cytometry analysis

Lymphocytes were isolated from the BF as previously described [19]. Monoclonal antibodies (mAbs) (CD3-SPRD, CD4-PE, CD8α-FITC) from Southern Biotech (Birmingham, USA) were employed. Cell suspensions were analyzed with a BD FACSCalibur Flow Cytometer (BD Biosciences, USA) and CellQuest software. The lymphocyte gate was defined by the forward/side scatter characteristics of the cells and 30,000 events were analyzed.

The mean values of the bursae from three PBS-inoculated unchallenged SPF chickens were used for normalization of the values of all experimental groups.

2.7. Histopathological analysis of the bursae

Bursal samples were placed in 10% neutral buffered formalin and paraffin embedded. Sections of BF were stained with haematoxylin and eosin following standard histological procedures and they were microscopically examined for the presence of bursal lesions under light microscopy. The scoring scale of histological damage is shown in Table 2.

2.8. IBDV isolation in CEFs

Viral isolation assays were performed using pieces of bursa that had been kept at −70 °C. They were mechanically disrupted in PBS and 3 frost/thaw cycles were performed. Homogenates were tenfold serially diluted and used to infect monolayers of CEFs seeded in 96-well plates as described in the seroneutralization assay. After 4 days at 37 °C, presence of CPE was determined. Viral titers were expressed as TCID50/gr, using the Reed and Muench method [20].

2.9. Statistical analysis

Statistical analysis was done with GraphPad Prism Software version 5.01. Means were compared using one-way ANOVA followed by multiple rank comparisons with Tukey's test. A probability (p) value of $\leqslant 0.05$ was considered statistically significant.

3. Results

3.1. Measurement of anti-IBDV and anti-VP2 humoral immune response

Results of the analysis of serum samples are listed in Table 2. Considering the manufacturer's cut-off point, the homologous pVP2 scheme induced the most consistent humoral immune response as all chickens immunized with this regime elicited antibody titers above the mentioned point. In regard to heterologous schemes, 5 over 6 chickens immunized with rVP2/pVP2 and 2 out of 6 animals receiving pVP2/rVP2 were positive for the test. All the chickens in both control groups were negative. The progression of antibody titers over time is shown in Supplementary Fig. 1.

When testing the sera for specific anti-VP2 antibodies (Fig. 1), all chickens immunized with either homologous or heterologous VP2 schemes had significantly higher antibody titers than both control groups ($p < 0.001$). Although none of the heterologous schemes were significantly different compared to the homologous pVP2 scheme, the group receiving rVP2/pVP2 had a significantly higher antibody titer than the one vaccinated with pVP2/rVP2 ($p < 0.05$). These results demonstrated that homologous or heterologous prime-boost immunization schemes were able to induce an IBDV specific humoral response. Besides, this immune response was influenced by the order in which rVP2 and pVP2 were inoculated in heterologous vaccination schemes.

3.2. Assessment of antibody neutralizing ability

To determine if the antibodies produced were capable of neutralizing the virus, a seroneutralization assay was performed. In this experiment, all groups immunized with schemes including VP2 were able to elicit significantly higher SN titers than both control groups (Fig. 2). While there were no significant differences between titers from homologous and heterologous VP2-schemes, the median SN titer of the group immunized twice with pVP2 was considerably higher than the ones from both heterologous schemes.

3.3. Evaluation of T cell infiltration in the BF after challenge

Increased infiltration of T lymphocytes into the BF has been associated with IBDV infection and replication in such organ [19,21]. Hence, the level of T cell infiltration in the bursa after challenge with IBDV can be taken as a parameter of vaccine protective efficacy. In this context, we analyzed the percentage of T $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ cells (Fig. 3) in the bursa of chickens challenged with a high dose of Winterfield strain of IBDV.

Regardless of the VP2-scheme received, all groups inoculated with this antigen had significantly less infiltration of T cells –both $CD4^+$ and $CD8^+$ – in their bursae than both control groups ($p < 0.001$). However, no significant differences were found between neither homologous vs. heterologous nor between heterologous schemes, showing that all VP2-schemes were effective to decrease T cell infiltration in BF.

3.4. Analysis of morphopathological changes in the bursae

The analysis of microscopic lesions produced by IBDV replication in the BF is a valid tool for measuring the infection severity in chickens [22]. Microscopic alterations include lymphoid necrosis, formation of cystic cavities, infiltration of heterophils and fibrosis [23]. Thus, a histopathological analysis was made to search for evidence of IBDV replication in chickens' bursae.

Histopathological scoring results are presented in Table 2. In agreement with the other results, all VP2-immunized

Table 2
Anti-IBDV antibodies, bursal damage and viral load.

Groups	IBD Ab[A]	Histopathological BF lesions[B]					IBDV viral load ($TCID_{50}$/gr)[C]
		Score 1	Score 2	Score 3	Score 4	Mean score ± SD	
pVP2	6/6	3	3	0	0	1.5 ± 0.5^{a}	$<1.26 * 10^3//^{a}$
rVP2/pVP2	5/6	3	3	0	0	1.5 ± 0.5^{a}	$<1.26 * 10^3//^{a}$
pVP2/rVP2	2/6	6	0	0	0	1.0^{a}	$<1.26 * 10^3//^{a}$
rGFP	0/6	0	0	2	4	3.67 ± 0.5^{b}	$4.08 \pm 1.1 * 10^5//^{b}$
pGFP	0/6	0	0	3	3	3.5 ± 0.5^{b}	$2.6 \pm 1.53 * 10^5//^{c}$

[A] IDEXX IBD results consider the cut-off point indicated by the manufacturer (396). Proportions shown represent the number of positive chickens over the total number of animals in each group (n = 6).

[B] Values indicate the number of chickens classified with certain histopathological score in each group. *Score 1*: unremarkable. *Score 2*: up to 30% of lymphocyte depletion (LD) in bursal follicles and intraepithelial cystic formation (CF). *Score 3*: 30–70% of LD and intrafollicular CF. *Score 4*: >70% of lymphocyte depletion, with follicular necrotic and/or fibrotic changes. The mean bursal score ± SD of each group is also shown. [a,b]Different letters along the scores indicate significant differences ($p < 0.001$).

[C] The viral load is expressed as the mean ± SD of the viral titers of each group, which were calculated as described by Reed & Muench [20]. Chickens with less than $1.26 * 10^3$ $TCID_{50}$/gr were considered negative for this test. Different letters along the values indicate significant differences between treatments ($p < 0.001$ for$^{a/b-c}$ and $p < 0.05$ for$^{b/c}$).

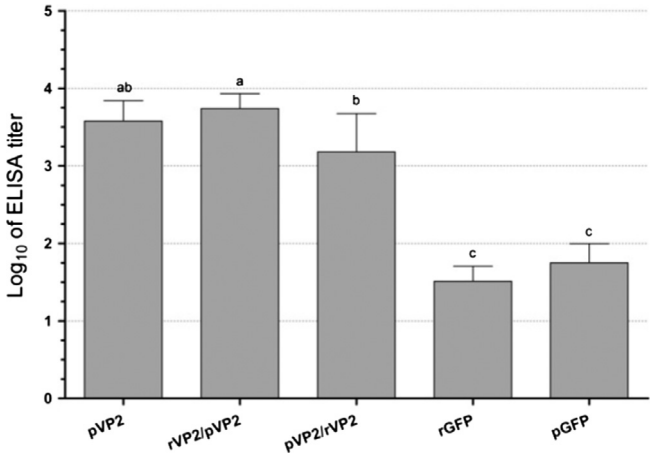

Fig. 1. Anti-VP2 antibody values evaluated through indirect ELISA. Sera from all chickens were tested just before challenge with IBDV. The antibody titers were Log10-transformed prior to analysis. Each column represents the mean value ± SD of the corresponding group. [a,b,c]Different letters above bars indicate significant differences among treatments ($p < 0.05$).

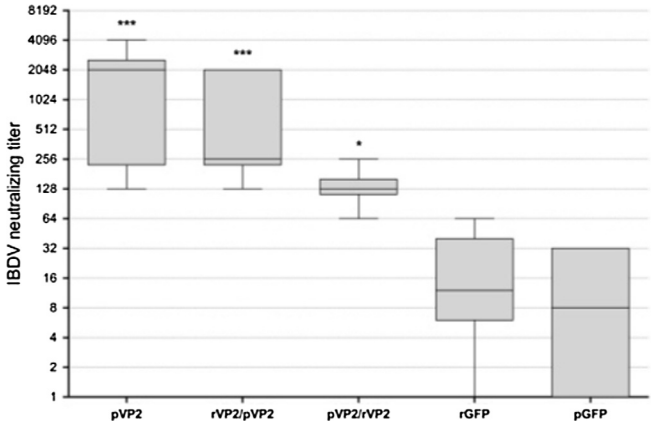

Fig. 2. Anti-IBDV neutralizing antibodies. A seroneutralization (SN) test was performed over the sera obtained just before challenge. The graph shows the median SN titer of each group (line inside the box) as well as the range of SN titers elicited. Asterisks above the boxes indicate significant differences between groups immunized with VP2 and both control groups ([***]$p < 0.001$ and [*]$p < 0.05$).

chickens had significantly lower scores than their counterpart GFP-immunized animals ($p < 0.001$), indicating that VP2 antigen prevented histological damage. Although no significant differences were found between the schemes with IBDV antigen, animals that received pVP2 as a prime and rVP2 as a boost were completely and homogeneously protected, whereas groups immunized with the inverse heterologous or the homologous pVP2 schemes had up to 50% of their animals showing higher degree of pathological changes.

Images of the morphopathological changes in the bursae are included in Fig. 4.

3.5. Quantification of IBDV viral load in BF

The quantity of IBDV in BF after challenge can be used as an indicator of the protective efficacy of an experimental immunogen. In our experiment, results showed significantly higher IBDV titers in both control groups ($p < 0.001$) compared to all groups immunized with either homologous or heterologous VP2 schemes (Table 2). Although there were no differences among the three evaluated immunization schemes, approximately 200-fold and 300-fold reductions of IBDV titer in BF were observed when comparing them with pGFP-group and rGFP-group, respectively.

4. Discussion

The use of heterologous prime-boost schemes has been widely explored in the last two decades. The key principles to support this rationale are the ability to elicit both humoral and cell-mediated immune responses and the avoidance of anti-vector immunity issues [12,24]. Recombinant poxviruses have been extensively tested in heterologous schemes and their effectiveness is the reason by which these vaccine vectors are currently among the most interesting candidates for HIV vaccines [25]. In mammals, recombinant MVA has been widely used as a booster in this kind of immunization protocols [26], showing better results when administered in that order [13]. Priming with DNA vaccines and boosting with non-replicative viral vectors has shown to be very effective to induce strong cell-mediated immunity [27]. On the other hand, either in mammals or chickens, proteic antigens are regarded as preferential stimulators of the humoral immune response and, thus, expected to elicit higher levels of antibodies than DNA or viral-vectored vaccines.

Successful experiences with heterologous prime-boost also exist in the avian model: DNA prime-inactivated vaccine boost [28–31], DNA prime-protein boost [32], recombinant Fowlpox prime-inactivated vaccine boost [33] and recombinant Adenovirus prime-recombinant MVA boost [16] have all shown to be more immunogenic when compared to homologous schemes.

In this context, we investigated: (a) whether a heterologous prime-boost immunization with a subunit VP2 vaccine and a recombinant MVA-VP2 could offer a higher anti-IBDV protective efficacy than the homologous prime-boost with the subunit VP2 and, (b) if the order in which immunogens were given could affect the immune response obtained.

As a general parameter of immunogenicity, anti-IBDV antibody titers were evaluated. In this assay, all the homologous pVP2 immunized animals were positive, whereas 5/6 animals immunized with rVP2/pVP2 scheme and 2/6 receiving the inverse heterologous scheme were positive. A more specific anti-VP2 response was then evaluated, showing significantly better results in rVP2/pVP2 immunized animals compared to those chickens receiving the inverse sequence of antigens. Although none of the heterologous schemes were significantly different from the homologous one, here we had the first evidence that the order of administration of the different antigens might be important.

In agreement with previous reports, pVP2 elicited high SN titers [8]. In contrast, repeated immunizations with rVP2 had not resulted in a strong humoral response [9] and, thus, it was possible that the combination of both immunogens elicited less SN antibodies than the homologous pVP2 scheme. However, when comparing heterologous schemes there was a tendency towards a better performance of rVP2/pVP2 over the inverse one. Altogether, these findings suggest that the order in which both immunogens are combined can affect the humoral immune response obtained.

Gao and coworkers [32] have reported that a protein boost after a DNA priming resulted in the highest anti-IBDV humoral response, and other researchers have reported similar results using DNA prime-inactivated vaccine boost [28] or viral vectored prime-inactivated vaccine boost [33]. Therefore, the rationale behind priming with a vectored antigen and boosting with a proteic one makes sense if what is sought is a strong humoral response while also stimulating cell-mediated immunity. As an additional advantage, it has been suggested that the smaller amount of antigen pro-

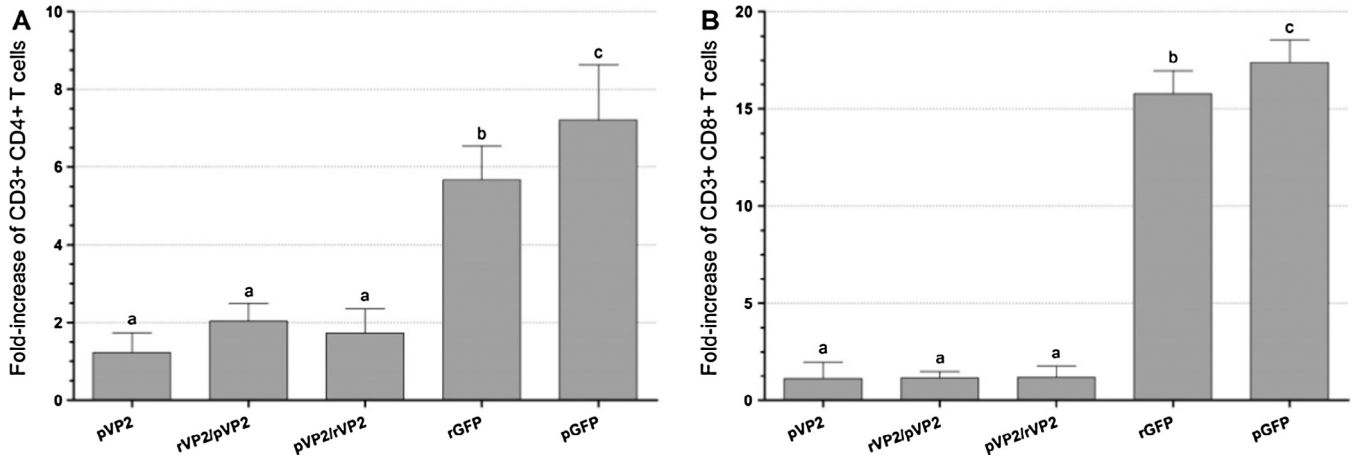

Fig. 3. Infiltration of T lymphocytes in the bursa of Fabricius after IBDV challenge. Chickens were challenged with Winterfield strain of IBDV and euthanized 5 days post challenge. T-cell subpopulations present in BF were stained with specific antibodies and analyzed by flow cytometry. All individual values were normalized with the mean percentage of each T-cell subtype obtained from healthy birds and then analyzed. Graphs show the mean fold-increase ± SD of the percentages of (A) T CD3+ CD4+ and (B) CD3+ CD8+ cells in each group. Different letters over the bars indicate significant differences among treatments ($p < 0.001$ for$^{a/b-c}$; $p < 0.05$ for$^{b/c}$).

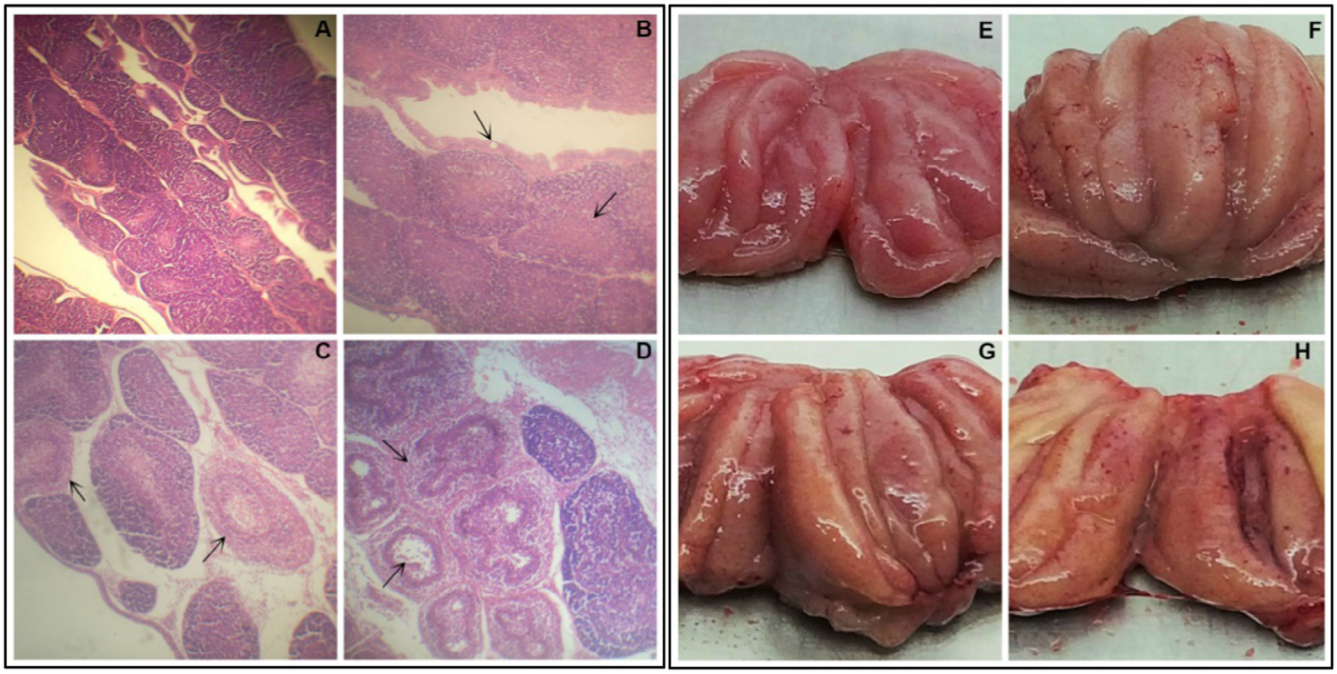

Fig. 4. Morphopathological changes of the bursae after IBDV challenge. An evaluation of pathological changes in the bursae was done at 5 days post challenge with the Winterfield strain of IBDV. The left panel shows H&E stained sections of bursae with score 1 (A), 2 (B), 3 (C) and 4 (D) in a 10× magnification. The arrows point to characteristic pathological changes as intraepithelial cysts and low degree of follicle lymphoid depletion primarily in the medullar area of follicles (B), high degree of lymphoid depletion affecting even the cortex of the follicles (C) and severe lymphoid depletion in many follicles (D). The right panel shows representative macroscopical pictures of bursae with score 1 (E), 2 (F), 3 (G) and 4 (H). A progression from rosy to a yellowish color can be seen. Also, an increment of haemorrhagic and edematous changes are observed within high-scoring bursae.

duced in the initial immunization in this kind of scheme could select for higher avidity in the antibodies produced [34].

Although neutralizing antibodies have been considered the most relevant tool to protect against IBDV infection, recent studies have raised doubt about the degree of dependence on them [35]. Some findings of our study support the hypothesis about the need of a more balanced immune response. When merging the results obtained in the analyses made on the bursae, we found that all the VP2-vaccinated animals had significantly less infiltration of T-cells ($p < 0.001$), IBDV viral loads ($p < 0.001$) and lower histopathological mean scores ($p < 0.001$) than the control groups. These results suggest that the three evaluated alternatives were able to prevent IBDV from reaching the BF or to enhance viral clearance from the affected tissue. However, an interesting finding of this study is that, despite not being significantly different, the histopathological scores were slightly better in the group primed with pVP2 and boosted with rVP2. This contrasts with the results of the humoral evaluation, although is consistent with other reports that found that less or even no detectable antibodies did not obliterate the ability of certain vaccine candidates to protect in both avian [11,36,37] and mammalian [38,39] models.

It should also be pointed out that T-cell-dependent viral clearance can induce bursal damage and delay follicle recovery [40], which may be the cause of mild histopathological damage in BF.

Therefore, it can be thought that more viral particles reached the bursa in those animals receiving homologous pVP2 or heterologous rVP2/pVP2 schemes compared to chickens immunized with pVP2/rVP2. However, further evaluations are needed to clarify the role of the antigen sequence over the slight differences in protection parameters.

Overall, the heterologous prime-boost approach did not show clear evidence of being better than the homologous one. However, the order in which the heterologous immunogens were delivered was able to influence over the immune response, as the rVP2/pVP2 scheme performed better when evaluated through humoral immunity parameters, while pVP2/rVP2 was slightly better preventing bursal damage. In a real field situation, an ideal prime against IBDV should be able to surpass maternal antibodies at an early stage – recombinant MVA may do this [41] and it has been successfully used for *in ovo* vaccination [16] – and the boost should be given with a highly immunogenic protein – as pVP2 – around 14 days after prime to achieve high levels of protection by the time when chicks become more susceptible to the infection. In spite of this, it remains unclear whether the humoral or the cellular branch of the immune response is the most important against IBDV, but this study is to be taken as a starting point to explore the mechanisms through which recombinant MVA can enhance anti-IBDV protective efficacy in the chicken.

Conflict of interest

The authors confirm that they have no conflict of interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.11.029.

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