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Plant-based vaccine candidate against Infectious bursal disease: An alternative to inactivated vaccines for breeder hens

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ABSTRACT

Infectious bursal disease (IBD) is an acute, highly contagious immunosuppressive disease that affects young birds causing important economic losses in the poultry industry worldwide. Strict hygiene management together with effective vaccination programs are the most important strategies to prevent Infectious bursal disease virus entry in poultry production facilities. Hyperimmunisation of dams with inactivated vaccines just before the laying period provides passive immunity to the progeny that protects them during the critical first few weeks after hatching before vaccination with live attenuated virus takes place. In the present study, a safe and economic plant-based vaccine candidate against IBD intended for breeder hens was evaluated. We demonstrated that the recombinant immunogen is effective as booster for previously primed hens since it increases specific antibodies against VP2 that are transmitted to the offspring with titres and decay rate similar to those achieved by inactivated vaccine. Moreover, these maternally derived antibodies have virus neutralising activity and are able to confer protection against challenge in progeny, as evidenced by absence of bursal damage and low viral titres in this organ. Taking into account the disadvantages of inactivated vaccines as well as the benefits of plants as expression systems, such as time and cost efficiency, lower risk of contamination from animal pathogens and nearly unlimited scalability, a plant-based subunit IBD vaccine represents a viable alternative in the veterinary field.

1. Introduction

Infectious bursal disease (IBD), also known as Gumboro disease, is an acute, highly contagious immunosuppressive disease that affects young birds worldwide. IBD causes important economic losses in the poultry industry both directly, through clinical signs and mortality, and indirectly, due to failure in vaccination programs and incremented susceptibility to other pathogens [1,2]. Its etiological agent is the Infectious bursal disease virus (IBDV), a non-enveloped icosahedral bisegmented double-stranded RNA virus, member of the *Birnaviridae* Family [3,4]. IBDV infects and destroys IgM bearing B-lymphocytes in the bursa of Fabricius (BF) causing severe immunodepression of B cell response in chickens [5]. The age and breed sensitivity of the birds, the virulence of the viral strain and the level of maternal antibodies constitute the main factors that will determine the outcome of an IBDV infection [6–9]. Although IBD affects birds mainly between 3 and 6 weeks of age, field exposure to IBDV in less than 3 week-old susceptible chicks can lead to subclinical infections which cause bursal atrophy and, in consequence, immunosuppression [10].

As there is no specific treatment for Gumboro, attention should be focused on preventive measures. Strict hygiene management together with effective immunisation programs are the most important strategies to prevent IBDV infection in poultry production facilities. Although vaccinations schemes may vary considerably between farms, they usually consist in the hyperimmunisation of breeder hens with inactivated vaccines just before the laying period in order to provide passive immunity to the progeny, and the inoculation of the offspring with live attenuated virus once the titre of maternally derived antibodies (MDA) has

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waned to non-protective levels [1,11]. However, conventional vaccines have a number of disadvantages due to their viral nature and there is a genuine need to replace them with new alternatives with higher efficacy and fewer side-effects. In particular, inactivated vaccines are costly due to the expense of propagating and inactivating the virus and require strict quality controls since improper inactivation could lead to disease outbreaks [12]. Moreover, they lack efficient immunogenicity unless they are combined with adjuvants and administered in repeated injections, or follow a prime with a replicating antigen [13]. For these reasons, their use is normally constrained to breeder birds that have been previously sensitised with live vaccine.

IBDV's capsid protein VP2, which contains the major neutralising epitopes, has been used for the development of subunit vaccines in a variety of heterologous systems (reviewed in 2,13,14). Our laboratory has developed a plant-derived vaccine candidate for IBD based on the transient expression of VP2 in *Nicotiana benthamiana* [15]. We have demonstrated that two intramuscular injections of the recombinant immunogen without adjuvant were enough to elicit a protective immune response in specific pathogen free (SPF) chickens with high titres of specific and neutralising antibodies. In addition, we observed that this immunisation scheme prevented the entrance or replication of IBDV in bursa, as evidenced by the low viral titres in this organ, as well as little T-cell infiltration and bursal damage [16].

Although subunit vaccines are safer than traditional IBDV vaccines, they are usually less immunogenic than live attenuated, viral-vectored or immune complex vaccines which can induce a strong immune response with only one dose in young birds. However, plant-derived VP2 might represent an attractive vaccine candidate for breeder hens before the laying period as an alternative to conventional inactivated vaccines. With this in mind, the objective of this work was to evaluate if our recombinant immunogen i) is able to induce a uniform long-lasting high titre serum antibody response in breeder hens and ii) provides passive immunity to the offspring by means of MDA capable of protecting chicks against IBDV infection.

2. Materials and methods

2.1. Genetic engineering of the expression vector.

The coding region of the mature VP2 (1323 bp) was amplified from the Argentinian field isolate LD-847-04 of IBDV (GenBank accession number for VP2 protein precursor gene: JF965438.1) kindly provided by Dr. Delamer (Laboratorio Delamer S.R.L., Argentina) and cloned into the pEAQ-HT vector developed by Sainsbury and Lomonossoff, John Innes Centre, UK [17]. The resulting expression vector was introduced into *A. tumefaciens* strain GV3101 by electroporation.

2.2. Transient expression of VP2

Transient expression was performed by infiltrating *Nicotiana benthamiana* leaves with a suspension of recombinant bacteria as previously described [18]. The infiltrated leaves were harvested 5 days postinoculation and blended with 3 volumes of chilled PBS with protease inhibitor cocktail (Roche, Mannheim, Germany). Leaf extract was centrifuged for 30 min at 20,000*g* and filtered through gauze. Samples were kept at −80 °C until use.

2.3. Detection and quantification of the recombinant protein

VP2 expression was analysed by western blot assays. Briefly, extracted proteins were separated in 12% SDS-PAGE and blotted onto nitrocellulose membrane. Proteins were identified using an anti-VP2 rabbit polyclonal antibody produced in our laboratory. Quantification of VP2 was estimated by comparison with a standard curve of bovine serum albumin (BSA). Briefly, serial two-fold dilutions of BSA from 250 μg/ml to 15.1 μg/ml were loaded and resolved in a 12% SDS-PAGE along with the sample of interest. After Coomassie Brilliant Blue staining, bands were analysed with Gel-Pro Analyzer software v3.1.

2.4. Experimental design

Two independent experiments were performed.

Experiment 1: Sixty 18-week-old Barred Plymouth Rock breeder hens that had been immunised at 4 and 7 weeks of age with live attenuated vaccine (Bursine 2®, Zoetis) were randomly divided into 3 treatment groups: No boost, VP2 and Inactivated vaccine. As indicated by its name, No boost group did not receive any additional inoculation while VP2 and Inactivated vaccine groups received an intramuscular injection of the recombinant immunogen (plant-crude extract containing 30 μg of VP2) or one dose of commercial killed vaccine (Gumboriffa, Boehringer Ingelheim Animal Health Argentina S.A). Blood was collected from each hen one week before vaccination and 3, 9 and 28 weeks post-boost (wpb). Specific antibodies against VP2 were analysed by in house ELISA. Twelve wpb, and following artificial insemination, eggs from all three groups were collected during a 3-day period and incubated separately until hatch in automatic incubators (Yonar, CABA, Argentina). Chicks were bled weekly during a month and specific IBDV antibodies in their sera were tested using a commercial ELISA.

Experiment 2: Forty-five 19-week-old Barred Plymouth Rock breeder hens that had been previously immunised with two doses of attenuated vaccine (Bursine 2®, Zoetis) were randomly divided into the same 3 groups described in experiment 1 and subjected to the same treatments. Specific anti-VP2 antibodies were analysed by in house ELISA from blood extracted from each hen one week before boost and 8 wpb. Following artificial insemination at 11 wpb, eggs were collected and incubated until hatch. Two weeks after hatching, 12 chicks from each group were bled to measure specific and neutralizing IBDV antibodies in their sera by commercial ELISA and seroneutralisation assay. Additionally, ten chicks per group were orally challenged with 10^4 ELD_{50} of a classical virulent Argentinian field strain isolated from broiler chickens in 2012 kindly provided by Dr Vagnozzi from Instituto de Virología, INTA, while 2 animals from each group remained unchallenged. One week later, chickens were euthanized and their bursas were removed to determine Bursal/Body Weight ratio (BB ratio), perform histopathological observation and quantification of viral load by RT-qPCR.

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).

2.5. Evaluation of humoral response

Sera obtained from hens were tested for specific anti-VP2 antibodies using an indirect ELISA based on IBDV subviral particles (SVP). Briefly, 96-well Maxisorp™ Nunc™ flat-bottom plates (Thermo Scientific, USA) were coated with 95 ng of SVP 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 a 1:400 dilution of sample sera, washed and incubated again with a 1:4000 dilution 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.

Sera obtained from chicks were evaluated for the presence of specific antibodies against IBDV with a commercial kit (cat No. 99-09260, IDEXX Laboratories, Inc., USA). Titres were calculated following the manufacturer's instructions. Values above 396 were considered positive.

2.6. Seroneutralisation assay

Seroneutralisation assay was performed as previously described with minor modifications [15]. Briefly, sera were inactivated for 30 min at 56 °C, serially diluted twofold in culture medium (50% MEM-D, 50% MEM-E, Hepes 1X, pH 7.4) and incubated with 100 $TCID_{50}$ of IBDV strain Winterfield for 1 h at 37 °C in 96-well plates. Subsequently, 100 μl of a cell suspension of 1×10^6 VERO cell/ml were added to each well. Cells were cultured at 37 °C, 5% CO_2 for 4 days, when cytopathic effect was observed. Neutralising antibody titres were calculated as the inverse of the last dilution showing no cytopathic effect.

2.7. BB ratio

Body weight and bursa weight were used to calculate the Bursa/Body Weight ratio according to the following formula: BB ratio = [bursa weight (g)/body weight (g)] × 1000.

2.8. Histopathological observation of bursa

Bursal samples were placed in 10% neutral buffered formalin and paraffin embedded. Sections of the paraffin embedded BF were stained with haematoxylin and eosin following standard histological procedures. The depletion degree of the lymphoid tissue in the bursas was evaluated by light microscopy and scored from 1 to 5, where 1 = normal BF, 2 = <25%, 3 = 25–50%, 4 = 50–75% and 5 = 75–100% of lymphoid depletion.

2.9. Viral load quantification in bursa

Total RNA was extracted from portions of bursa stored in TransZol (TransGen Biotech, Beijing, China) according to the protocol provided by the supplier. The quantity and quality of the extracted RNA was determined using NanoDrop™ ND-1000 (Thermo Scientific, Wilmington, USA) and agarose gel electrophoresis. cDNA synthesis and qPCR was performed in a single step reaction utilizing Luna® Universal Probe One-Step RT-qPCR Kit (New England Biolabs, Massachusetts, USA) according to the manufacturer's protocol. Primers used for retrotrancription and amplification were VP1f: 5′CCAACACACCTCATGATCTC3′ and VP1r: 5′GTCAATTGAGTACCACGTGTT3′ that amplify a product of 300 bp belonging to the VP1 protein of IBDV. Number of viral copies per microgram of RNA was calculated by extrapolation with a standard curve generated by qPCR from ten-fold dilutions of a plasmid containing the amplified VP1 fragment ranging from 10^2 to 10^9 copies number.

2.10. Statistical analysis

Statistical Analysis was performed using one way ANOVA and mean differences were analyzed with the Tukey test. The Shapiro-Wilk and Levene tests were applied to verify the assumptions. In the cases where heterocedasticity was detected, the variance structure was modeled and the best model was selected by AIC. Transformation of data was also performed when normality was not assumed. The Fischer Test was applied for comparing percentages. Also, Kruskal-Wallis and Dunńs Test were performed.

All the analyses were done with R [19] and GraphPad Prism Software version 5.01.

3. Results

Before performing chicken experiments, expression of recombinant VP2 in plant extracts was confirmed by Western blot and quantified by SDS-PAGE followed by Coomassie Brilliant Blue staining. A specific band corresponding to the mature VP2 was observed at the expected size and the estimated concentration of VP2 antigen in the plant extract was approximately 60 μg/ml (Fig. 1).

3.1. Humoral response in hens

To evaluate the ability of the recombinant immunogen to boost humoral response in previously primed birds, 18 week-old vaccinated hens were randomly divided into 3 groups. One group did not receive any boost (No boost group), while the other two groups were intramuscularly injected with either 500 μl of plant extract containing recombinant VP2 or one dose of commercial killed vaccine (VP2 and Inactivated vaccine groups respectively). Sera from individual hens were analysed for specific antibodies against VP2 using an in house ELISA. Fig. 2 shows the mean antibody level

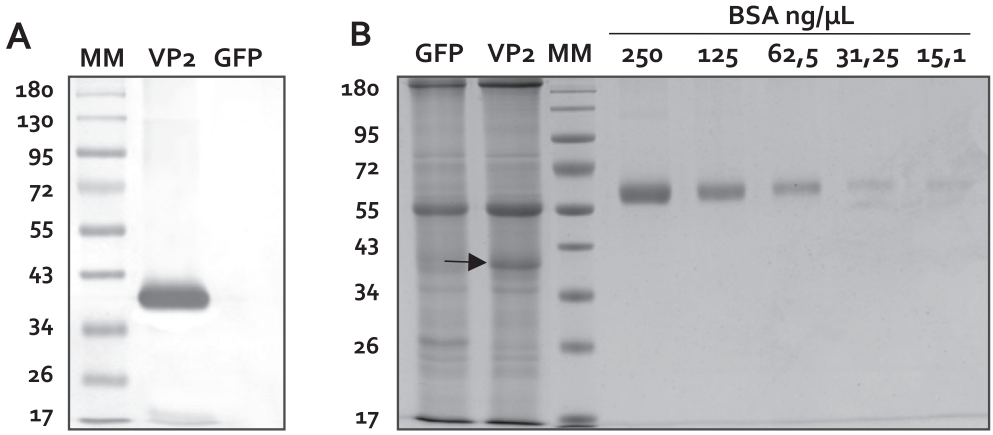


Fig. 1. VP2 transient expression in *Nicotiana benthamiana* plants. Extracted proteins from VP2 or GFP (negative control) agroinfiltrated leaves were separated on a 12% SDS-PAGE. (A) Identification of recombinant VP2 by western blot using an anti-VP2 antiserum. (B) Quantification of VP2 by comparison with a standard curve of bovine serum albumin (BSA) after Coomassie Brilliant Blue staining. MM: molecular marker.

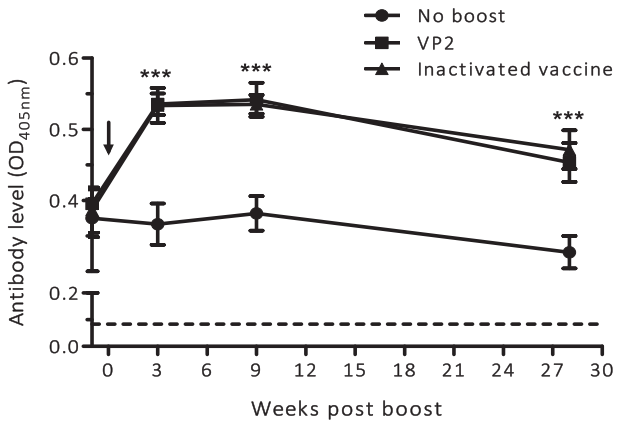


Fig. 2. Evaluation of anti-VP2 response in hens serum by in house ELISA: Specific antibody levels in sera of breeder hens unboosted or injected with VP2 or inactivated vaccine at day 0 (arrow) were measured over a six and a half-month period. Anti-VP2 levels are represented as the mean OD_{405} ± S.D. for each group at different points of the time course. Dotted line shows the absorbance of a negative serum. Significant differences between No boost and vaccinated groups were observed for all times evaluated (one-way ANOVA with Tukey post hoc test, $^{***}p < 0.001$).

(OD_{405nm}) obtained from each group through time. Before boost, all animals had anti-VP2 antibodies in their sera due to previous vaccinations with live attenuated vaccine and antibody levels were similar among groups. Both VP2 and inactivated vaccine were able to significantly increase specific antibody levels in sera three weeks after boost. Moreover, these antibodies remained elevated during several weeks and, although a decrease was observed by the 28th week, they were always significantly greater than No boost group throughout the 6–7 month period. No differences were detected between the recombinant immunogen and the commercial vaccine in any of the time points evaluated, demonstrating that they are equally effective at boosting specific humoral response in primed hens.

3.2. Maternal antibodies in offspring

To establish if induced anti-IBDV antibodies in breeder hens were transferred to the progeny, 30 to 40 chicks from each group were weekly bled during a one-month period. A commercial ELISA kit that allows us to obtain antibody titres in a standardized manner was used. Fig. 3A shows the percentage of chicks within each group that were considered positive for specific anti-IBDV antibodies during the times evaluated, while Fig. 3B shows the individual titres of positive chicks.

At day 7, the majority of the chicks from the three groups were positive for the presence of anti-IBDV antibodies (85, 91 y 97% for No boost, VP2 and Inactivated vaccine group respectively). However, when comparing antibody levels, No boost group titres resulted significantly lower than titres from the other two groups. At day 14, only eight animals out of 30 from No boost group had specific antibodies while 23 and 25 chicks of VP2 and Inactivated vaccine groups respectively, resulted positive. Just as observed on day 7, titres in these two groups were significantly higher than control group. By day 21, most of the antibodies had waned below cut-off values in all groups, with the exception of antibodies in three VP2 and six Inactivated vaccine animals. All chicks were seronegative at day 28. In none of the time points evaluated the percentage of positive animals or MDA titres presented significant differences between inoculated groups. These results demonstrate that maternally derived antibodies from hens boosted with the

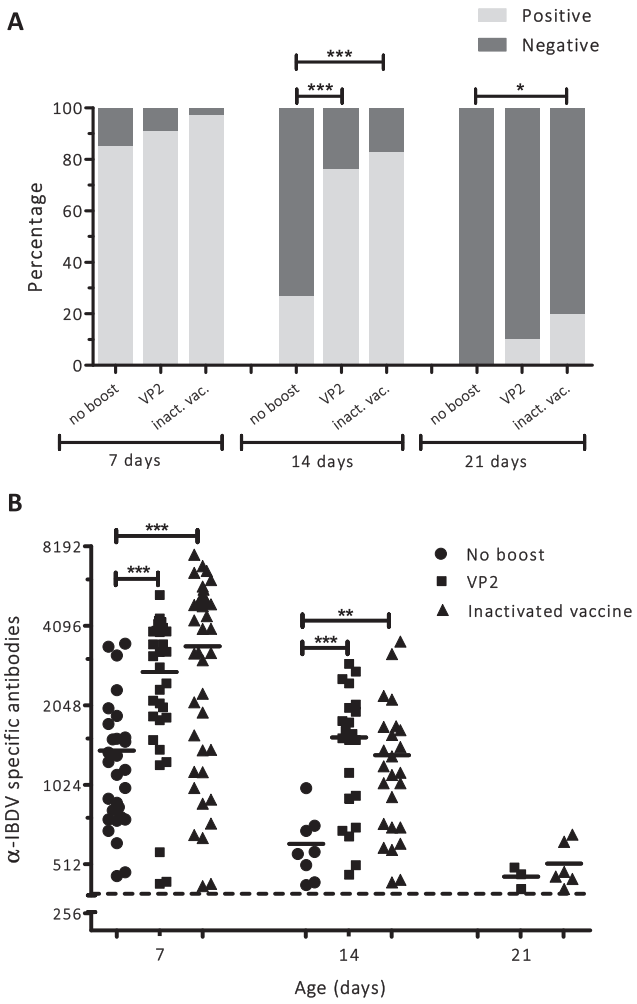


Fig. 3. Evaluation of anti-IBDV MDA in chick's serum by ELISA. Maternally derived antibodies in sera of chicks hatched from hens unboosted or injected with VP2 or inactivated vaccine were weekly measured by commercial ELISA. (A) Percentage of positive chicks for anti-IBDV antibodies according to manufacturer's instructions (titres > 396) (Fisher Test, $^{***}p < 0.001$, $^{*}p < 0.05$). (B) Individual antibody titres and mean (black line) of positive chicks in each group for the different time points was evaluated (one-way ANOVA with Tukey post hoc test, $^{***}p < 0.001$, $^{**}p < 0.01$).

recombinant immunogen or the inactivated vaccine display equivalent titres and kinetics in the offspring.

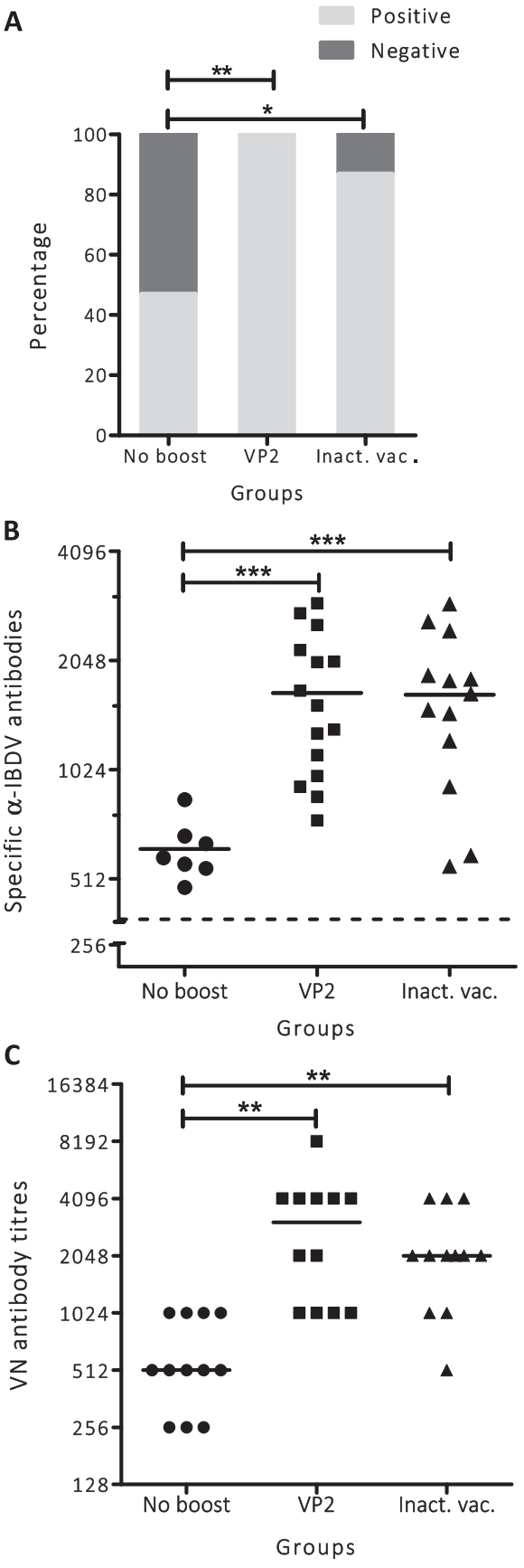
3.3. Passive protection against IBDV

In order to determine if the passive immunity transmitted by dams inoculated with the plant-derived VP2 was able to protect their offspring from IBDV infection, a second experiment was performed with a different set of animals.

Similarly to the first experiment, 45 previously vaccinated hens were randomly divided into three groups at 19 weeks of age and subjected to the same treatments. Anti-VP2 levels (OD_{405nm}) were measured in hens' sera before and 8 weeks after boost to confirm that immunisations had successfully boosted specific humoral response (Supplementary Fig. 1).

Ten 14-day-old chicks hatched from each group were orally infected with 10^4 ELD_{50} of a classical virulent strain field isolate, while two animals per group were left unchallenged to serve as healthy controls. This time was chosen for viral challenge since, as seen in the previous experiment and other reports, maternal antibodies are still high at 2 weeks of age [6,10,20,21]. Nonetheless, chicks were bled before challenge to determine the antibody

level in sera at the time of infection and to perform seroneutralisation assays. One week later, chickens were sacrificed and different parameters in bursa were analysed in order to establish protection.



Once again, percentage of positive animals and specific antibody titres in chicks hatched from inoculated groups were indistinguishable among them and significantly greater than No boost group (Fig. 4A and B). Although a slightly higher percentage of positive animals were seen in each group with respect to the first experiment, antibody titres were very similar in both experiments at day 14. Regarding virus neutralising (VN) antibodies, all groups presented MDA capable of neutralising IBDV infection in VERO cell culture compared to negative control serums (titres < 4, data not shown), even those animals which resulted negative for specific antibodies in the commercial ELISA (Fig. 4C). Still, VN titres from No boost were significantly lower than boosted groups and no significant differences were observed between VP2 and Inactivated vaccine.

To determine the protective efficacy of MDA against viral challenge we first estimated the BB ratio which is an established parameter for measuring bursal atrophy, a typical sign of IBDV infection. No significant differences were observed between groups and compared to unchallenged animals due to the high heterogeneity of the values (Table 1). However, the lowest BB ratios (<3.3) belonged to four No boost chicks while in the rest of the groups, no animals with ratios below 4 were found. Despite not being able to observe differences in this parameter, when analysing bursal scores to determine pathological lesions, No boost group proved to be more affected than boosted groups after infection (Table 1). All VP2 and 9 out of 10 of Inactivated vaccine animals had normal healthy bursas comparable to unchallenged ones. On the contrary, almost all chicks from No boost group had moderate to severe lymphoid depletion (>50% of affected tissue) with high levels of inflammatory infiltrate. Different degrees of edema, fibrosis and necrosis, as well as epithelial and follicular cysts, were observed in these bursas.

Finally, viral load in bursas was quantified by RT-qPCR in order to determine if IBDV was able to reach and/or replicate in its target organ. Fig. 5 shows the number of IBDV copies per microgram of bursal RNA in each animal. The sensitivity of the quantitative RT-PCR assay was estimated at 20 copies/200 ng of input RNA. While low viral loads were observed in bursas from Inactivated vaccine group (mean = 439 ± 271 #copies/μg RNA), no viral genome was detected in VP2 bursal samples. As expected, most of No boost chicks had elevated IBDV titres (as high as 2.5×10^7 #copies/μg RNA) with the exception of two animals that had comparable amounts to Inactivated vaccine group.

Together, these results demonstrate that MDA from hens inoculated with the recombinant immunogen have IBDV neutralising activity and are able to protect young chicks from viral infection. This passive immunity is as effective as the one achieved by conventional inactivated vaccines.

4. Discussion

IBDV was identified for the first time more than 50 years ago; still, this virus remains a significant threat to commercial poultry worldwide. Because of its stability in the environment, control through sanitation is important but often insufficient. This makes

Fig. 4. Evaluation of anti-IBDV MDA in chick's serum by ELISA. Maternally derived antibodies in sera of 14-day-old chicks hatched from hens unboosted or injected with VP2 or inactivated vaccine were measured by commercial ELISA and their virus neutralizing activity was assessed by a seroneutralisation test in VERO cell culture. (A) Percentage of positive chicks for anti-IBDV antibodies according to manufacturer's instructions (titers > 396) (Fisher Test, $^{**}p < 0.01$, $^{*}p < 0.05$). (B) Individual antibody titres and mean (black line) of positive chicks (one-way ANOVA with Tukey post hoc test, $^{***}p < 0.001$). (C) Individual virus neutralising antibody titres, calculated as the inverse of the last dilution showing no cytopathic effect, and median for each group (black line) (Kruskall-Wallis with Dunn post hoc test, $^{**}p < 0.01$).

Table 1
Response of chicks to IBDV challenge. (A) Number of animals within each bursal lesion score and median score of each group. Scores were assigned according to the degree of lymphoid depletion: 1 = normal BF, 2 = <25%, 3 = 25–50%, 4 = 50–75% and 5 = 75–100% of lymphoid depletion. Values within a column followed by different lowercase superscript letters are significantly different (Kruskall-Wallis test, $p < 0.0001$). (B) Bursa/Body Weight ratio calculated as BB ratio = [bursa weight (g)/body weight (g)] × 1000.

Group	Bursal lymphoid depletion						BB ratio
	Score 1	Score 2	Score 3	Score 4	Score 5	Median score	
Unchallenged	6	0	0	0	0	1[a]	6.15 ± 1.37
No boost	1	0	1	5	3	4[b]	5.00 ± 2.11
VP2	10	0	0	0	0	1[a]	6.13 ± 0.87
Inactivated vaccine	9	0	1	0	0	1[a]	5.48 ± 0.95

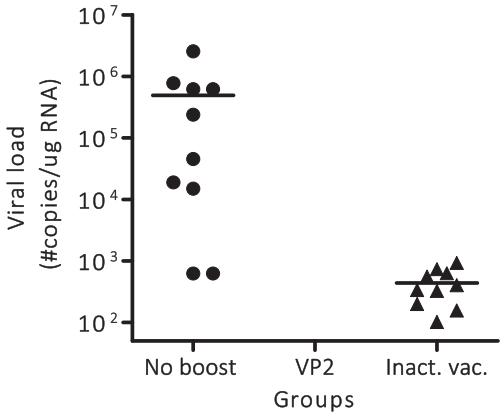


Fig. 5. Viral load in bursa 7 days post challenge. Number of viral copies/μg of bursal RNA were estimated by RT-qPCR. Individual viral titres and the mean (black line) for each group is shown. No viral genome was detected in VP2 group or unchallenged animals (data not shown). The sensitivity of the quantitative RT-PCR assay was estimated at 20 copies/200 ng of input RNA.

the vaccination of the flocks the most important action to prevent IBDV entry into any poultry production.

During the first few weeks after hatching, young chicks are most susceptible to the immunodepressive effects of the disease [22,23]. Since their immune system is not fully developed, specific maternal antibodies transferred from hens via the egg are the primary means of antigen-specific protection [24]. The duration of the parental immunity is variable in progeny and is dependent on the antibody levels in the dams [24,25]. Hence, the development of a high, uniform and long-lasting antibody response in breeder hens which can be transmitted to the offspring and protect them during the critical period before vaccination is of utmost importance.

In the present study, a plant-based vaccine candidate intended for the hyperimmunisation of breeder hens against IBDV was successfully evaluated. Although reports in which recombinant subunit vaccines were assessed as alternatives to traditional IBDV inactivated vaccines for hens are scarce, both polyprotein and VP2 have been expressed in yeast and baculovirus and used to immunise dams [26–28]. These recombinant immunogens formulated with Freund adjuvant were able to boost IBDV antibody titres or generate a *de novo* specific humoral response in primed [26,28] or unprimed hens [27]. Moreover, maternal antibodies were transferred to progeny and were able to protect chicks against IBDV infection as evidenced by the absence of viral antigen in bursa. Additionally, a bivalent vaccine consisting of inactivated Newcastle disease virus (NDV) and yeast-produced VP2 (Gumbin®VP2 Phibro Animal Health Corporation) intended for previously sensitised birds with live vaccines against ND and IBD actually reached the market demonstrating the feasibility of adoption of a subunit vaccine in the veterinary field. In this work, transient expression of VP2 in *Nicothiana benthamiana* was our system of choice for the development of the new vaccine candidate since plants present a number of benefits for antigen expression such as time and cost efficiency, lower risk of contamination from animal pathogens and nearly unlimited scalability. Furthermore, the use of plants as bioreactors to obtain simple and complex molecules has been well documented proving them to be suitable platforms for the production of human and veterinary vaccines [29,30].

When inoculated into previously vaccinated dams, recombinant immunogen (plant crude extract containing 30 μg of VP2) and whole inactivated commercial vaccine demonstrated to be equally effective in boosting specific anti-VP2 antibodies within three weeks of injection and these levels were significantly higher than those of unboosted hens throughout the period evaluated (28 weeks). These results are consistent with earlier reports were primed breeder hens boosted with 45 μg of yeast-produced VP2 [26] or 50 μg of baculovirus-derived VP2 (rVP2) [28] developed high specific antibody titres within 2 weeks after boost and remained elevated during at least 10–11 weeks.

In addition, chicks hatched from VP2 inoculated dams were found to have elevated levels of maternal anti-IBDV antibodies in their blood that exhibited similar titres and decay rate than MDA transferred from Inactivated vaccine group. Although at one week of age, most of chicks hatched from non-boosted hens were also considered positive for the presence of anti-IBDV antibodies, these had significantly lower titres than the other two groups. This was not surprising given the existing correlation between parental flock antibody levels and MDA transferred [21,31]. By week two, antibodies in more than half of No boost chicks had dropped to negative levels while a high percentage of the progeny from boosted groups still had elevated titres as shown in the two independent experiments. In spite of this, all sera evaluated in the seroneutralisation assay had virus neutralizing antibodies at day 14, even the ones that tested negative in the ELISA. Still, VN titres in boosted groups were significantly higher than No boost group. According to World Organization for Animal Health (OIE) when testing for the decay of maternally derived antibodies, it is not uncommon to find residual VN antibodies at an age when ELISA results are already negative [32]. Specific anti-IBDV antibodies disappeared by day 21 in almost all chicks with very few exceptions in VP2 and Inactivated vaccine groups. The MDA kinetics observed in progeny of the boosted dams concurs with numerous reports that describe the regression of maternal antibodies in hiperimmunised breeder hen's offspring [6,10,20,28,33]. Although in this work only the time course of total anti-IBDV antibodies was assessed, it would be interesting to carry out the same evaluation with VN antibodies.

It is important to highlight that passive immunity transmitted to VP2 chicks was able to protect them against IBDV challenge with 10^4 ELD_{50} of a virulent field isolate as demonstrated by a variety of parameters. All bursas from this group were completely healthy and indistinguishable from bursas of unchallenged animals. Furthermore, no virus was detected in this organ by RT-qPCR. Although only one chick from the Inactivated vaccine group showed moderate lymphoid depletion, in 9/10 animals IBDV was detected in bursas suggesting that our recombinant immunogen was more effective in preventing viral entrance and/or replication

in its target organ. On the contrary, No boost group, with the exception of one animal, was severely affected by IBDV infection, as evidenced by bursal lesions and high viral load, despite the presence of residual VN in antibodies in their serum.

Although it has been reported that the maternal antibody titres in progeny chicks are demonstrable up to the 3rd, sometimes even the 4th week of age, the protective limit of these antibody levels usually expires by the second week [6]. However, since the extent of the protection depends on the initial titre of IBDV in chicks, which is a direct reflection of the immune status against IBD in the parental flock, and vaccinations programs vary considerably between farms, it is common to find diverse results in the literature. Ahmed and Akhter (2000) showed that chicks from broiler breeders with known IBDV vaccination history were completely protected from experimental challenge with virulent IBDV up to 14 days of age while mortality varied from 0 to 20% between day 14 and 35 although MDA persisted until the 21th day. In another study, chicks hatched from fertile eggs obtained from three different broiler breeder flocks with different levels of IBDV neutralizing antibodies were challenged at different weeks of age [10]. Chicks with medium levels of MDA were protected from infection at 1 and 2 weeks of age while protection in chicks with high titres lasted until the 4th week. It is worth mentioning that the VN titres observed in our No boost group were higher than the ones that conferred protection to the Medium and High Antibody groups at day 14 and 28 respectively, yet, they were not able to prevent infection as seen by Al-Natour et al. [10]. A possible explanation could be the difference in the strain and the larger dose of virus used for challenge in this work (10^4 ELD_{50} of a virulent strain vs 10^2 ELD_{50} of a variant strain). It would be of interest to evaluate the extent of the passive protection elicited by our recombinant immunogen through the execution of viral challenges in chicks hatched from VP2 vaccinated dams with a variety of strains and doses at different days of age.

Here, we demonstrated that VP2 transient expression in *Nicothiana benthamiana* represents a viable platform for the production of a safe, economic and efficacious vaccine against Infectious bursal disease which would be beneficial to the poultry industry. Furthermore, no formulation of the recombinant immunogen with additional adjuvants was needed in order for it to evoke a potent and uniform immune response which would lower the costs of the final product even more compared to traditional inactivated vaccines. As discussed previously [16], this may be due to plant components present in the crude extract used for the immunisations (e.g. saponins, polysaccharides, lectins) which may help modulate the immune response. It is yet to be determined whether, or to what extent, certain degree of purification to get rid of *Nicotiana benthamiana* alkaloids is required and how will the loss of plant components affect the immunogenicity of the vaccine candidate. There are numerous established protocols for purifying biopharmaceuticals proteins and vaccine antigens from tobacco, but they are usually laborious and expensive since they aim at very high protein purity levels [34,35]. Purification is necessary for human intended products but might not be essential for the veterinary field. Recently, Stephan et al. (2018) described a simple and inexpensive purification method that provided a high recovery yield of purified colicins from *N. benthamiana*, as well as a drastic reduction of nicotine to levels that could enable the final products to be used on food. This purification method could be applicable to other cost-sensitive proteins such as veterinary antigens [36]. Other options are to use *Nicotiana* spp. plants with low content of alkaloids [37] or to use plant systems free of alkaloids, such as *Lactuca sativa* (lettuce), however the efficacy of the crude extract as immunogen must be tested. Altogether, the results obtained in this work suggest that our plant-based subunit IBD vaccine candidate represents a viable alternative to the inactivated vaccines traditionally administered to breeder hens before the laying period.

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All authors attest they meet the ICMJE criteria for authorship.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.07.069.

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