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Study of coinfection with local strains of infectious bursal disease virus and infectious bronchitis virus in specific pathogen-free chickens

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ABSTRACT Immunosuppressive diseases cause great losses in the poultry industry, increasing the susceptibility to infections by other pathogens and promoting a suboptimal response to vaccination. Among them, infectious bursal disease virus (**IBDV**) arises as one of the most important around the world. IBDV infects immature B lymphocytes, affecting the immune status of birds and facilitating infections by other pathogens such as avian infectious bronchitis virus (**IBV**). Although it has been reported that the interaction between these viruses increases IBV clinical signs, there are no actual studies about the interaction between regional circulating isolates that validate this statement. In this context, the objective of our work was to evaluate the effect of the interaction between local isolates of IBDV (belonging to genogroup 4) and IBV (lineage GI-16) in chickens. Thus, specific pathogen-free chickens were orally inoculated with IBDV genogroup (**G**) 4 or with PBS at 5 d of age. At 14-days postinoculation (**dpi**) the animals were intratracheally inoculated with a GI-16 IBV or with PBS. At multiple time points, groups of birds were euthanized and different parameters such as histological damage, viral load, lymphocyte populations and specific antibodies were evaluated. The success of IBDV infection was confirmed by the severity of bursal atrophy, viral detection, and presence of anti-IBDV antibodies. In IBV-infected animals, the presence of viral genome was detected in both kidney and bursa. The coinfected animals showed higher degree of lymphocyte infiltration in kidney, higher rate of animals with IBV viral genome in bursa at 28 dpi, and a clear decrease in antibody response against IBV at 28, 35, and 40 dpi. The results indicate that the infection with the local isolate of IBDV affects the immune status of the chickens, causing major severe damage, in response to IBV infection, which could consequently severely affect the local poultry industry.

Key words: coinfection, IBDV, IBV, specific pathogen-free chicken, local strain

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INTRODUCTION

Infectious bursal disease virus (**IBDV**) is a nonenveloped virus with a segmented double-stranded RNA genome belonging to the *Birnaviridae* family. It is capable of generating humoral immunosuppression against other pathogens but, curiously, not against itself (Skeeles et al., 1979; Azad et al., 1985; Sharma et al., 2000). It is known that the younger the animals infected with IBDV, the greater the immunosuppression (Faragher et al., 1974; Saif, 1991). Traditionally, IBDV has been classified in antigenic terms as classic, variant and distinct. Variant and distinct strains that can evade the protection provided by vaccines formulated with classical strains causing severe atrophy of the bursa of Fabricius have been isolated in various parts of the world (Jackwood and Saif, 1987; Snyder et al., 1988; Heine et al., 1991; Sapats and Ignajatovic, 2000; Yamazaki et al., 2017). Particularly, strains known as distinct have been reported in Argentina, Eastern Asia, Eastern Europe, Brazil, Canada, and Uruguay (Domanska et al., 2004; Ojkic et al., 2007; Hernández et al., 2015; Vera et al., 2015; Yamazaki et al., 2017; de Fraga et al., 2019). In Argentina, it has been reported that most of the isolates obtained between 2005 and 2012, from animals with clinical signs, corresponded to distinct strains named at first "Argentinean lineage" and reclassified later as isolates belonging to genogroup 4 (**G4**) (Vera et al., 2015; Michel

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and Jackwood, 2017). It has been suggested that the high prevalence of these isolates in Argentinean poultry farms could be due to the evasion of the immune response induced by vaccines based on classical strains (Vera et al., 2015). It has been described that isolates from genogroup 4 can be pathogenic even for animals that were vaccinated with classical vaccines, although mortality was not reported (Jeon et al., 2009; Yamazaki et al., 2017; Tomás et al., 2019). Consequently, it could be relevant to evaluate the degree of immunosuppression generated by G4 IBDV and its effect on the coinfection with the local IBV variant.

Besides, infectious bronchitis virus (**IBV**) is an enveloped virus with a positive single-stranded RNA genome belonging to the *Coronaviridae* family (De Groot et al., 2012). This virus replicates in respiratory, reproductive, kidney, and digestive tissues (Cavanagh et al., 1992). The most recent classification proposed by Valastro and coworkers consists of 6 main genotypes (GI−GVI) and 32 viral lineages (1−32) (Valastro et al., 2016). It is considered a relevant avian viral pathogen that causes great losses in the poultry industry after avian influenza virus and Newcastle disease virus (OIE, 2017). Despite extensive vaccination programs against avian bronchitis (IB), it remains a threat in South America and throughout the world (Alvarado et al., 2005; Rimondi et al., 2009; Marandino et al., 2017). IBV strains belonging to GI-1 (vaccine strains), GI-11, and GI-16 have been reported to be present in Argentinian flocks for long time, being GI-11 located exclusively in South America (Marandino et al., 2017; Marandino et al., 2021; Marandino et al., 2022).

In 2013, high mortality episodes were reported in broiler chickens in Argentina (>15%), and isolates belonging to GI-16 were repeatedly detected (Gerez et al., 2021). In addition, it has been suggested that a local strain called A13 isolated from Argentinean flocks, belonging to GI-16, can replicate in animals subjected to different vaccination schedules (Gerez et al., 2021).

It has been reported that IBV infection can be aggravated by previous infections with IBDV (Pejkovski et al., 1979). Moreover, animals vaccinated with an immune-complex IBDV vaccine and subsequently vaccinated with an attenuated IBV vaccine were found to have altered immunological parameters compared to animals vaccinated with a recombinant vaccine (Lupini et al., 2020).

In order to gain insights into the pathogenicity of local viral strains and to test whether the immunosuppression elicited by IBDV could explain the IBV outbreaks, we performed an experiment of 2 serial infections with both viruses and measured infectivity parameters.

MATERIALS AND METHODS

Experimental Chickens

Embryonated eggs laid by specific pathogen-free (**SPF**) White Leghorn hens were purchased from Instituto Rosenbusch S.A. (Buenos Aires, Argentina) and hatched in an automatic incubator (Yonar, Buenos Aires, Argentina). Chickens were kept in individual cages with food and water ad libitum. All procedures were performed in agreement with institutional guidelines and approved by the Institutional Animal Care and Use Committee (CICUAE-13/2019).

Viruses

The infectious bursal disease virus [1/chicken/ARG/P33/15 (P33)], belonging to genogroup 4 (GenBank accession number MN313610.1) and the infectious bronchitis virus [AR/13/BA/A13 (A13)], belonging to genogroup I-16 (GenBank accession number MH718950.1), were both isolated from commercial establishments (Marandino et al., 2019; Tomás et al., 2019). These viruses were amplified in embryonated White Leghorn SPF eggs and viral stocks, obtained after 5 passages, were kept at −80°C and titrated as previously described (Jaton et al., 2022).

Experimental Procedure

Fifty-six White Leghorn SPF chickens were randomly divided into 4 groups. One group ($n = 14$) was inoculated with 10^2 ELD_{50} of IBDV at 5 d of age and with sterile phosphate-buffered saline (**PBS**) at 14-days postinoculation (**dpi**) (IBDV group). Another group ($n = 14$) was inoculated with PBS at 5 d of age and with 2×10^5 ELD_{50} of IBV at 14 dpi (IBV group). A third group ($n = 14$) received 10^2 ELD_{50} of IBDV at 5 d of age and 2×10^5 ELD_{50} of IBV at 14 dpi by the oral route (IBDV/IBV group). Finally, the control group ($n = 14$) received PBS at 5 and 19 d. Four or 5 chickens were sacrificed at 21- and 28-days post first inoculation. The remaining 5 chickens were kept until d 42 pi for clinical observation and serological evaluation. Food (Metrive S.A., Buenos Aires, Argentina) and water were provided ad libitum.

Clinical Signs, Gross Analysis, and Sample Processing

Chickens were monitored daily for any anomaly. We looked for typical signs of IBDV and IBV such as depression, watery diarrhea, ruffled feathers, and dehydration. In addition, in the case of IBV, we looked for the presence of respiratory signs. At different time points, chickens were euthanized and postmortem examinations were carried out to evaluate body and bursa weight and histopathological changes. In addition, bursa, kidney, and spleen were harvested, observed for macroscopic lesions, weighed, and cut into several pieces. One piece of bursa and kidney was submerged in TransZol solution (TransGen Biotech, Beijing, China) for RNA extraction. Another piece of bursa and spleen were submerged in RPMI culture medium (Gibco, Grand Island, NY) for lymphocyte isolation, and the remaining piece was submerged in 10% formalin for histopathological analysis.

Sera were collected at 14, 21, 28, 35, and 40 dpi for the detection of anti-IBDV and anti-IBV specific antibodies.

Bursa to Body Weight Ratio

Body and bursa weights were used to calculate the bursa to body weight ratio (**BB** ratio) according to the following formula: BB ratio = [bursa weight (g)/body weight (g)] × 1,000 (Raji et al., 2017).

Histopathological Analysis

Bursa and kidney samples were submerged in 10% neutral buffered formalin and paraffin-embedded. Sections were stained with hematoxylin and eosin following standard histological procedures and they were microscopically examined for the presence of lesions under light microscopy. Table 1 shows bursal lesions and the weighting factor (**WF**) assigned to them according to the importance and compromise of the organ functionality. The severity was determined by evaluating each lesion in 5 fields at 100× and giving a lesion score (**LS**) value from 0 to 4, where 0 = normal BF, 1 = <25%, 2 = 25 to 50%, 3 = 50 to 75%, and 4 = 75 to 100% of affected tissue. Then, the bursal score (**BS**) was calculated using the formula:

$$\mathrm{BS} = \sum \mathrm{Lesion\ Score(LS)} \times \mathrm{Weighting\ Factor(WF)}$$

In the kidney, we evaluated inflammatory cell infiltration (II) and hemorrhage (H). The severity was determined by evaluating each lesion in 5 fields at 100× and scoring them from 1 to 4, where 1 = normal, 2 = <33%, 3 = 33 to 66%, and 4 = 66 to 100% of affected tissue.

Humoral Immune Response

Sera obtained from chickens were tested for specific anti-IBDV antibodies using an indirect ELISA based on IBDV subviral particles (**SVP**) developed in our laboratory (Gómez et al., 2020). Briefly, 96-well Maxisorp Nunc flat-bottom plates (Thermo Scientific Inc., Waltham, MA) 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 1:4,000 dilution of goat anti-chicken IgG antibodies coupled to horseradish peroxidase (Bethyl Laboratories Inc., Montgomery, TX). The revealing step was performed using ABTS substrate (Sigma-Aldrich, St. Louis, MO)-H_2O_2 in citric acid buffer, pH 5. Reading was done at 405 nm after 20 min of incubation. Results were expressed as percentage of positivity (**PP**) using the formula:

$$\mathrm{PP} = [(\mathrm{A405nmS} - \mathrm{A405nmNC})/(\mathrm{A405nmPC} - \mathrm{A405nmNC})] \times 100,$$

where PC is the positive control; NC is the negative control; and S is the sample.

In addition, sera obtained from chickens were tested by duplicate for specific antibodies against IBV using an indirect ELISA developed in our laboratory. Briefly, IBV antigen was obtained by infection of embryonated eggs and subsequent collection of allantoic fluid and purification by sucrose gradient. The antigen was diluted in 50 mM carbonate/bicarbonate buffer pH 9.6 at a concentration of 300 ng/well and used to coat 96-well plates (Thermo Scientific), which were incubated for 16 h at 4°C and then washed 3 times with PBS-T. Subsequently, plates were blocked with 150 μL/well of PBS-T 5% adult equine serum (**AES**) + 5% normal goat serum (**NGS**), for 1 h at 37°C. Once the blocking solution was discarded, serum samples were added at a 1/100 dilution in PBS-T 5% AES + 5% NGS and incubated for 1 h at 37°C with shaking. After 3 washes with PBS-T, a commercial anti-chicken IgG antibody labeled with peroxidase (Bethyl Laboratories) was added to the plates at a 1/10,000 dilution in PBS-T 5% AES + 5% NGS. Plates were incubated for 1 h at 37°C with shaking. Subsequently, they were washed 3 times with PBS-T and the development of the assay was carried out with TMB (Sigma-Aldrich). The reaction was stopped with 2N H_2SO_4 after 7 min. Optical density (**OD**) values were recorded with an automatic Multiskan EX microplate reader (Thermo Labsystems) at 450 nm.

Results were expressed as PP using the formula:

$$\mathrm{PP} = [(\mathrm{A450nmS} - \mathrm{A450nmNC})/(\mathrm{A450nmPC} - \mathrm{A450nmNC})] \times 100,$$

where PC is the positive control; NC is the negative control; and S is the sample.

Table 1. Weighting factors (WF) of histopathological lesions in bursa.

Lesion	WF assigned
Lymphoid depletion (LD)	5
Fibrosis (F)	4
Inflammatory cell infiltration (II)	3
Edema (E)	4
Necrosis (N)	4
Intraepithelial cysts (IEC)	1
Intrafollicular cysts (IFC)	5

Flow Cytometry

Mononuclear cells were isolated from bursal and spleen samples and used to study T and B cell populations by flow cytometry as previously described (Jaton et al., 2022). Briefly, bursae were cut into very small pieces and mechanically disrupted by pressing with a syringe plunger, in RPMI 1640. Then, cellular suspensions were passed through a 40 μm mesh (Cell Strainer, BD), and mononuclear cells were isolated by centrifugation over a Histopaque density gradient (1.077 g/mL; Sigma, St. Louis, MO) at room temperature. Cells were recovered from the interface, washed, and live cells were counted using trypan blue exclusion.

Then, cells were diluted in staining buffer and 1×10^6 cells per well were seeded on 96 well plates (V-shape) and washed twice with the same buffer. Staining was performed by resuspending cells in different combinations of antibodies, or as single-color stainings for compensation. Cells were incubated at 4°C for 30 min and washed twice with staining buffer. Monoclonal antibodies (**mAbs**) anti-chicken: CD4-PE, CD8a-FITC, TCR$\gamma\delta$-PE, and IgM-FITC were purchased from Southern Biotech (Birmingham, AL). All the antibodies were titrated to determine the optimal staining concentration of each one. Positive cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software, LLC. Lymphocyte gates were defined by the forward/side scatter characteristics of the cells and 30,000 events were analyzed for each sample.

Viral Load

Total RNA was extracted from pieces of bursa and kidney and stored in TransZol (TransGen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. The quantity and quality of the extracted RNA were determined using a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE) and agarose gel electrophoresis. The complementary DNA (**cDNA**) synthesis and qPCR were performed in a single-step reaction using Luna Universal Probe One-Step RT-qPCR Kit (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. Primers used for retrotranscription and amplification of IBV were 216-f: 5′TACA TCCGTTGCTTGGGCTA3′ and 490r: 5′ACCAGAA CCTGTCACCTC3′ which amplifies a 275 bp product belonging to the IBV genome starting from position 216 to position 491. Primers used for retrotranscription and amplification of IBDV were VP1f: 5′CCAACACACC TCATGATCTC3′ and VP1r: 5′GTCAATTGAGTAC CACGTGTT3′ that amplify a product of 222 bp belonging to VP1 gene. The number of viral copies per microgram of RNA was calculated by extrapolation with a standard curve generated by qPCR.

Statistical Analysis

One-way ANOVA was used to perform statistical analyses and mean differences were analyzed with the Tukey test. The Shapiro-Wilk and Levene tests were applied to verify the assumptions. Transformation of data was also applied when normality was not assumed. When assumptions were not fulfilled, Kruskal-Wallis nonparametric test or Wilcoxon nonparametric was applied followed by Wilcoxon pairwise comparison. The P value was set to <0.05 for the statistical tests. All the analyses were done using R 3.4.1 (R core team) and agricolae package (De Mendiburu, 2014).

RESULTS

Bursa to Body Weight Ratio

Chickens were infected with a local isolate of IBDV G4 and subsequently (14 d later) with a local isolate of IBV GI-16, to determine if IBDV infection altered IBV pathogenicity. No clinical signs were observed along the experiment.

To determine the degree of bursal atrophy in IBDV-infected animals, the BB ratio was measured at 21, 28, and 42 dpi with IBDV ($N = 5$, $N = 4$, and $N = 5$, respectively). Figure 1 shows that all animals infected with IBDV presented significantly lower BB ratios than non−IBDV-infected animals; furthermore, no differences were observed between IBDV and IBDV/IBV groups (2-way ANOVA test and Tukey post hoc test, $P < 0.05$). In addition, the BB ratio remained altered up to 42-days postinfection, indicating that animals could

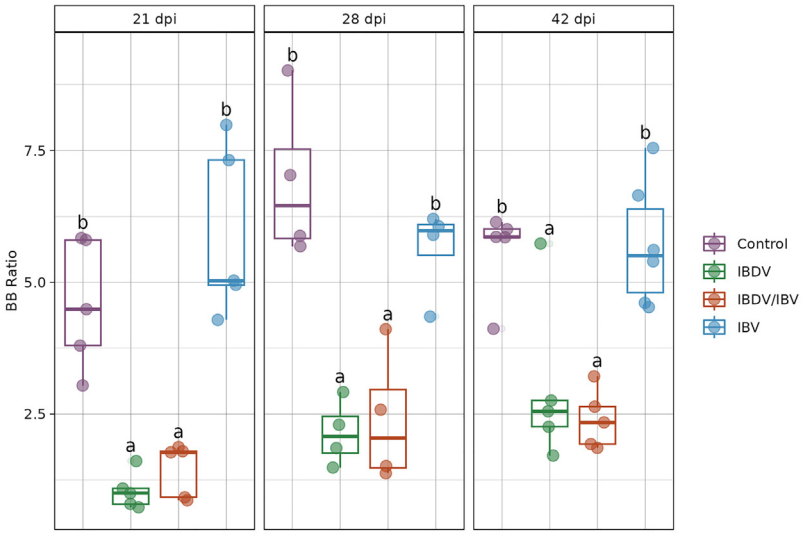

Figure 1. Bursa to body weight ratios of Control, IBDV, IBDV/IBV, and IBV groups. Chickens were sacrificed and weighed at different postinfection time points. Bursectomy were performed and bursae were weighted. Individual BB ratios were determined by the formula [bursa weight (g)/body weight (g)] × 1,000. The box plots represent data distribution. Different letters indicate significant differences among groups (2-way ANOVA test and Tukey post hoc test, $P < 0.05$).

not recover the normal size of the bursa during the time of the experiment.

Histopathological Observation of Bursa and Kidney

Samples of bursa and kidney were taken to determine histological damage on each time point. The lesions evaluated in bursa included: lymphoid depletion (**LD**), fibrosis (**F**), inflammatory cell infiltration (**II**), edema (**E**), necrosis (**N**), intraepithelial cysts (**IEC**), and intrafollicular cysts (**IFC**). In the kidney, inflammatory infiltrate (typical IBV lesion) and hemorrhage were observed. The Figure 2A shows that those chickens inoculated with IBDV presented characteristic lesions of infection at 21, 28, and 42 dpi. Thus, only groups infected with IBDV are shown, since the others did not present any type of bursa lesion. The maximum damage degree was registered at 21 dpi; then it decreased and

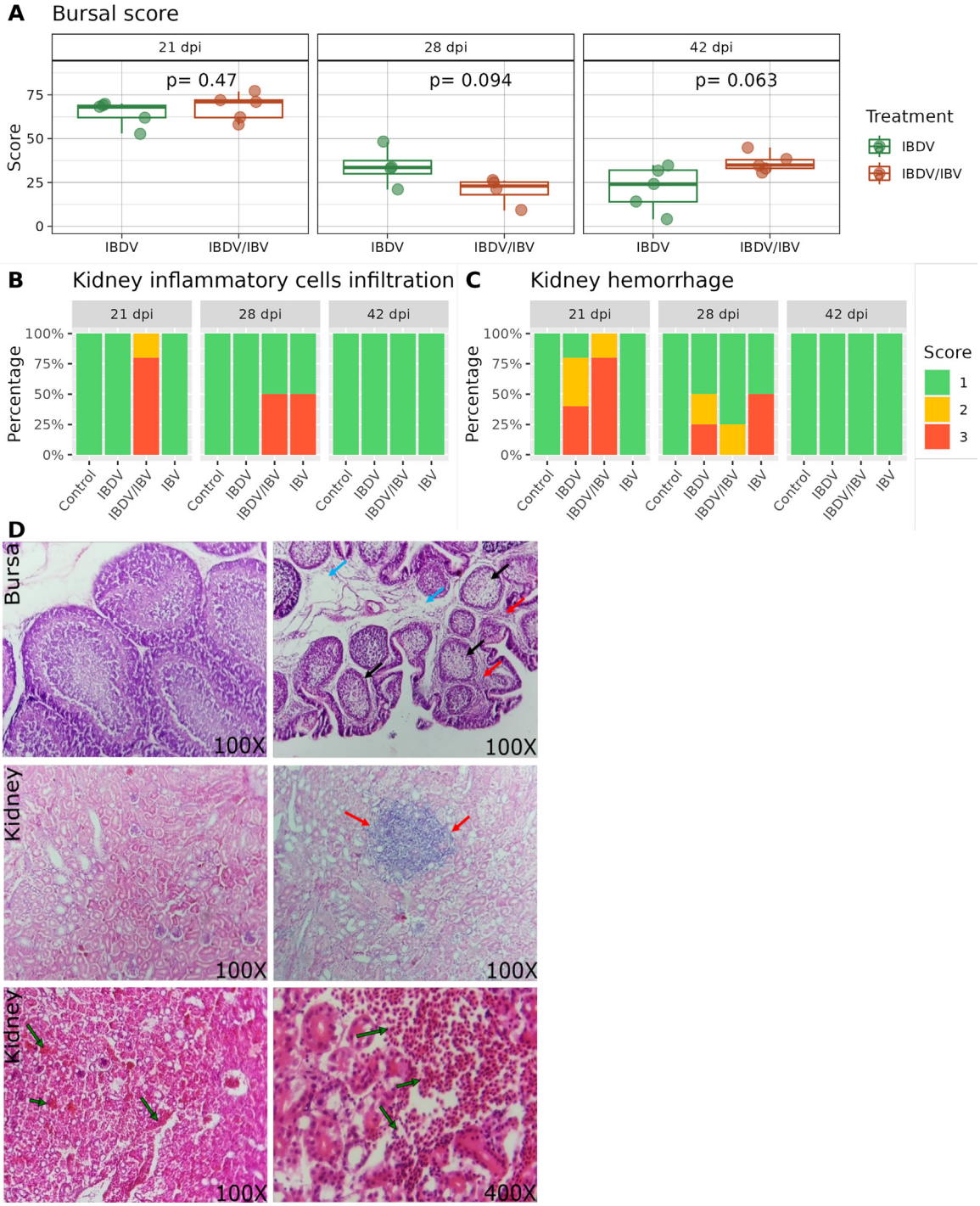

Figure 2. Histopathological evaluation of bursa and kidney. Bursae and kidneys extracted at 21, 28, and 42 dpi, were fixed and stained for histological evaluation. (A) Bursal score of IBDV and IBDV/IBV groups. The P value is shown for each day (t test). (B and C) Proportion of chickens with differences in score of inflammatory infiltrate (B) and hemorrhage (C) in the kidney. Colors indicate the severity of the lesion. (D) Representative photographs of the most typical bursal and renal lesions. Lymphoid depletion (black arrow), inflammatory infiltrate (red arrow), edema (light blue arrow), and hemorrhage (green arrow).

remained low until 42 dpi. Figure 2B and C shows that groups inoculated with IBDV presented differences on kidney scores at 21 and 28 dpi. Particularly, IBDV/IBV group was the only one that showed lesions at 21 dpi when analyzing the inflammatory infiltrate; however, on d 28 pi, no differences were detected between IBDV and IBDV/IBV groups (Figure 2B). Regarding kidney hemorrhage, IBDV/IBV group presented the highest number of animals with severe degree of injury on d 21 pi. On the other hand, no major differences were observed between infected groups at 28 dpi, showing that both viruses can produce hemorrhage in kidney (Figure 2C). Both kidney parameters, hemorrhage and lymphocytic infiltrate, were recovered at 42 dpi. Figure 2D shows the most typical lesions found in bursa and kidney.

Humoral Immune Response

Antibody response against IBDV and IBV was determined by ELISA. Results were expressed as the PP. All chickens belonging to the groups that were inoculated with IBDV presented PP above 20% (from 14 to 40 dpi), proving the infection was successful (Figure 3A). Besides, the kinetics of antibodies against IBV was determined. Figure 3B shows that at 28 dpi (14 d after IBV infection) the percentage of positivity of anti-IBV antibodies was significantly decreased in chickens coinfected with IBDV in comparison to animals that had been infected only with IBV, remaining lower until the end of the experience (Wilcoxon test: $^*P < 0.05$).

Flow Cytometry

At 21 and 28 IBV dpi, mononuclear cells from bursa and kidney were isolated and labeled with different staining panels. Thirty thousand events from each sample were analyzed and results are expressed as a percentage of populations and subpopulations in side and forward scatter viable lymphocyte gate.

No differences were observed in lymphocyte populations proportions in bursa of IBDV-, IBDV/IBV-, and IBV-infected animals in comparison to control group, being Bu+ B cells >95% of total bursal cells (data not shown). When analyzing spleen, we observed some alterations in lymphocyte populations (Figure 4). Differences in CD8+ cells between IBDV and control and between IBDV/IBV and IBV groups were observed at 21 dpi, being animals infected with IBDV the ones that showed higher percentage of CD8+ cells, while CD4+ cells showed no significant differences in the frequency between groups in any analyzed days. The analysis of TCR$\gamma\delta$+ cells showed that only IBDV/IBV presented significant differences with control and IBV groups at 21 dpi, while both IBDV and IBDV/IBV had a significantly higher percentage of these cells in spleen than the control at 28 dpi. All statistical analyses were conducted using ANOVA test and Tukey's post hoc test, $P < 0.05$.

Viral Load

To determine how coinfection affects IBV viral replication, we evaluated differences in IBV viral load by RT qPCR in kidney and bursa of chickens infected with IBV or with IBDV/IBV.

We found no significant differences in kidney or in bursa at the time points between both groups, indicating that previous infection with IBDV did not alter IBV replication. No IBDV viral load was detected in bursa at 28 dpi in animals from IBV group.

In addition, we quantified the viral genome in IBDV and IBDV/IBV groups, in order to determine if the infection with IBV altered the viral load of IBDV in the bursa. We found no significant differences between groups, despite the coinfected animals presented higher viral load at 21 dpi. Notably, viral loads were extremely low at both times, probably due to sampling time (Figure 5).

DISCUSSION

Immunosuppression caused by IBDV is a major concern in the poultry industry. Previous research has

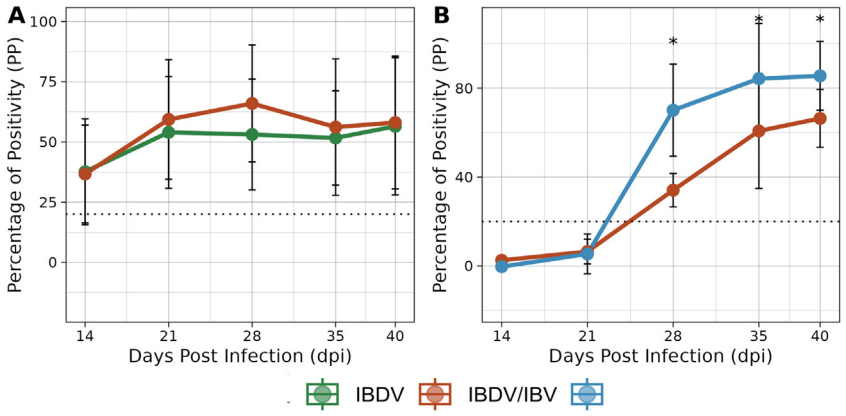

Figure 3. Anti-IBDV and anti-IBV humoral immune response. The percentage of positivity (PP) of anti-IBDV (A) and anti-IBV (B) antibodies was determined at 14, 21, 28, 35, and 40 dpi with an indirect ELISA specific for IBDV or IBV, and the mean PP values ± SD are shown for each group at these time points; data from the IBV and Control (A) and IBDV and Control (B) were less than 20% (data not shown). Significant differences between IBDV/IBV and IBV groups were observed at 28, 35, and 40 dpi (Wilcoxon test: $^*P < 0.05$).

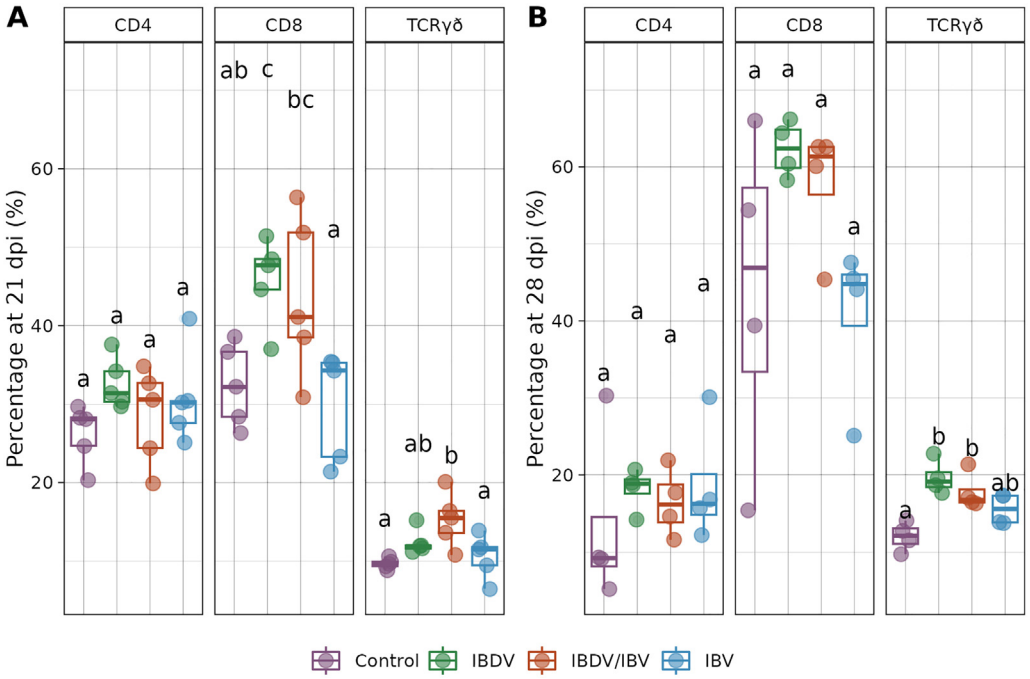

Figure 4. Analysis of lymphocyte populations of spleen by flow cytometry. Mononuclear cells were isolated from spleen, stained with different combinations of antibodies, and analyzed by flow cytometry. The lymphocyte population was gated according to size and complexity. The results are shown as the percentage of cells in each analyzed group. (A) Percentage of CD4+, CD8, and TCRgd at 21 dpi. (B) Percentage of CD4+, CD8+, and TCRgd at 28 dpi. Different letters indicate significant differences among groups (2-way ANOVA test and Tukey post hoc test, $P < 0.05$).

shown that the severity of the immunodepression is higher in young chickens, leading to complications with other pathogens (Faragher et al., 1974; Saif, 1991; van den Berg et al., 2000; Gimeno and Schat, 2018; Tomás et al., 2019). Although maternal antibodies and vaccines may provide some initial protection, some viral variants can still evade immunity and cause infection in young animals (Yamazaki et al., 2017). The previous infection with IBDV can intensify the pathogenicity of many morbid agents, such as IBV (Pejkovski et al., 1979). In 2013, an outbreak of IBV in Argentina caused a mortality rate above 15% (Gerez et al., 2021). To better understand the pathogenicity of local isolates and to investigate whether the immunosuppression caused by an IBDV isolate could explain in some extent the 2013 IBV outbreak, we performed an experiment involving 2

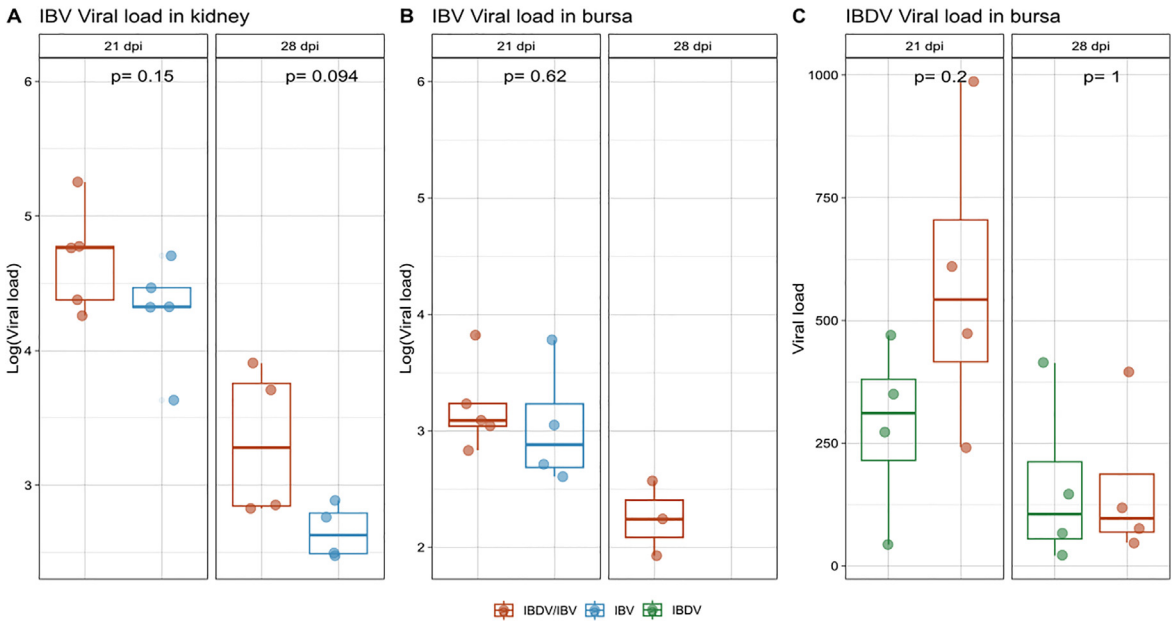

Figure 5. Detection of IBV and IBDV viral load. IBV was detected in kidney (A) and bursa (B) and IBDV viral load was measured I bursa (C). Kidney and bursa were collected at 21 and 28 dpi and total RNA was extracted. The number of IBV and IBDV viral copies/μg total RNA was estimated by RT-qPCR. Individual logarithmic values (dots), as well as box plots representing data distribution, are show for each group. The P value is shown for each day (t test).

serial infections with both viruses, measuring infectivity parameters.

Usually, IBDV causes histological damage and reduction in bursal size at the age of susceptibility (3−6-wk old), but the effects on these parameters at earlier ages of birds are not well known. During the analysis of histological damage in bursa and reduction of the BB ratio we observed that the strain 1/chicken/ARG/P33/15 was able to generate severe atrophy and damage of bursa indicating that this strain had similar pathotype behavior as other isolates of the same genogroup in SPF chickens (Yamazaki et al., 2017; Tomás et al., 2019).

We found a significant reduction of anti-IBV antibody titers from 14 d after IBV inoculation, indicating that our IBDV isolate partially affected humoral response against IBV. Similar results were reported by Lupini et al. using an immunocomplex IBDV vaccine at 1 d of age and an attenuated IBV vaccine 14 d later (Lupini et al., 2020). Although the authors did not consider the cut-off point suggested by the commercial kit (ELISA-IDEXX), they reported significant differences in antibody titers at 14 and 21 d after IBV vaccination in animals that had been previously vaccinated against IBD. Tomás and colleagues described similar results using a strain belonging to G4 (Tomás et al., 2019). Animals infected at 1 d of age with 1/chicken/URY/1302/16 and vaccinated 15 d later with a live NDV vaccine showed significant decrease in antibody titers 15 d after vaccination in comparison to the uninfected group (Tomás et al., 2019).

The local isolate of IBV A13 used in the present study can replicate in kidneys of infected animals (Gerez et al., 2021). Therefore, we analyzed the lesions observed in that tissue (Collisson et al., 2000; Lisowska et al., 2021). We found that only those chickens previously infected with IBDV showed a high lymphocytic infiltrate score at 21 dpi (7-days post-IBV inoculation), while no differences were evidenced at 28 dpi. Conspicuously, these results did not correlate with a decrease in viral load at 28 dpi, since it has already been shown that lymphocytic infiltration is responsible for viral clearance (Collison et al., 2000). Similar results were observed by Xu et al. when studying the response of chickens coinfected with IBDV and a fowl adenovirus, finding that the coinfected group showed the highest score up to 7 dpi (Xu et al., 2021). These results might indicate that a previous infection with IBDV has an impact on the acute phase of IBV infection, allowing a faster innate immune response in the target organ. On the other hand, the presence of hemorrhage was detected only in IBDV-infected animals and at 21 dpi. The ability of IBDV to cause hemorrhage in the kidney has been previously described elsewhere (Ley et al., 1983; Singh et al., 2015). On the other hand, several studies have shown that IBV infection can produce hemorrhage in kidney at 7 dpi or earlier (El-Bahrawy et al., 2017; Najimudeen et al., 2022). In this work, we describe for the first time the ability of A13 isolate to cause kidney hemorrhage.

When analyzing cell populations by flow cytometry we observed low BB ratios along with high histological damage in the bursa of IBDV-infected chickens. The frequency of lymphocyte populations did not show differences to control chickens, being mainly Bu+ cells (data not shown). Interestingly, Lupini and colleagues reported similar results when analyzing lymphocyte populations in the spleen, Harderian gland and trachea (Lupini et al., 2020). It has been described that several IBDV isolates produce lymphoid depletion during the first days after infection that could be reversed later (Kim et al., 1999; Jaton et al., 2022). Although lymphocyte populations were able to recover after IBDV infection at the evaluated time points, it cannot be stated that the bursa fully recovers its function, which was suggested by the reduced ability of animals in the IBDV/IBV group to produce antibodies against IBV.

The analysis of IBV viral load in kidney did not reveal significant differences among groups at 21 dpi. However, at 28 dpi, a trend toward higher viral load was observed in IBDV/IBV treated chickens compared to IBV-infected ones. It is noteworthy that IBV isolates are known to be able to replicate in the bursa (Ambali and Jones, 1990). Nevertheless, at 28 dpi, although viral loads were extremely low, 3 out of 5 animals in the IBDV/IBV group tested positive, while none in the IBV group.

This result suggests that the previous infection with IBDV may delay (in some way) the IBV clearance, contributing to a longer viral detection in the bursa. It has been shown that feces containing IBV is capable of infecting other animals (De Wit, 2000). Therefore, it is possible to assume that by remaining a longer time in the intestinal tract, the viral spreading period is consequently lengthened.

This study shows that there are differences between chickens infected with both IBDV and IBV when compared with those infected only with IBV. Given that laboratory conditions are more controlled than those found in poultry farms (de Wit and Cook, 2014), it is likely that coinfection in commercial farms might result in a more severe problem and higher losses for poultry production than those reported in this study. Our data support the importance of implementing the necessary sanitary measures to minimize the risk of coinfection of the flocks with these 2 avian viral pathogens.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that

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REFERENCES

Alvarado, I. R., P. Villegas, N. Mossos, and M. W. Jackwood. 2005. Molecular characterization of avian infectious bronchitis virus strains isolated in Colombia during 2003. Avian Dis. 49:494–499.

Ambali, A. G., and R. C. Jones. 1990. Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus. Avian Dis. 34:809–817.

Azad, A. A., S. A. Barrett, and K. J. Fahey. 1985. The characterization and molecular cloning of the double-stranded RNA genome of an Australian strain of infectious bursal disease virus. Virology 143:35–44.

Cavanagh, D., P. J. Davis, J. K. Cook, D. Li, A. Kant, and G. Koch. 1992. Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. Avian Pathol. 21:33–43.

Collisson, E. W., J. Pei, J. Dzielawa, and S. H. Seo. 2000. Cytotoxic T lymphocytes are critical in the control of infectious bronchitis virus in poultry. Dev. Comp. Immunol. 24:187–200.

de Fraga, A. P., T. Gräf, V. P. Coltro, N. Ikuta, A. S. K. Fonseca, N. Majó, and V. R. Lunge. 2019. Phylodynamic analyses of Brazilian antigenic variants of infectious bursal disease virus. Infect. Genet. Evol. 73:159–166.

De Mendiburu, F. 2014. Agricolae: statistical procedures for agricultural research. R Package Version 1. Lima, Perú.

De Groot, R. J., S. C. Baker, R. Baric, L. Enjuanes, A. E. Gorbalenya, K. V. Holmes, S. Perlman, L. Poon, P. J. M. Rottier, P. J. Talbot, P. C. Y. Woo, and J. Ziebuhr. 2012. Family Coronaviridae. Pages 806−828 in Virus taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. A. M. Q. King, M. J. Adams, E. B. Carstens and E. J. Lefkowitz, eds. Academic Press, San Diego, CA, USA.

De Wit, J. J. 2000. Detection of infectious bronchitis virus. Avian Pathol. 29:71–93.

De Wit, J. J. S., and J. K. A. Cook. 2014. Factors influencing the outcome of infectious bronchitis vaccination and challenge experiments. Avian Pathol. 43:485–497.

Domanska, K., T. Mato, G. Rivallan, K. Smietanka, Z. Minta, C. de Boisseson, D. Toquin, B. Lomniczi, V. Palya, and N. Eterradossi. 2004. Antigenic and genetic diversity of early European isolates of infectious bursal disease virus prior to the emergence of the very virulent viruses: early European epidemiology of infectious bursal disease virus revisited? Arch. Virol. 149:465–480.

El-Bahrawy, A., A. Zaid, Y. Sunden, M. Sakurai, H. Ito, T. Ito, and T. Morita. 2017. Pathogenesis of renal lesions in chickens after experimental infection with 9a5b Newcastle disease virus mutant isolate. Vet. Pathol. 54:94–98.

Faragher, J. T., W. H. Allan, and P. J. Wyeth. 1974. Immunosuppressive effect of infectious bursal agent on vaccination against Newcastle disease. Vet. Rec. 95:385–388.

Gerez, R., A. Marandino, G. Tomas, V. Olivera, S. Pinto, M. I. Craig, C. Techera, R. Perez, and A. Vagnozzi. 2021. Evaluation of the efficiency of commercial vaccines against infectious bronchitis virus (IBV) belonging to the GI-16 lineage isolated in an Argentinean outbreak. Avian Dis. 65:456–462.

Gimeno, I. M., and K. A. Schat. 2018. Virus-induced immunosuppression in chickens. Avian Dis. 62:272–285.

Gómez, E., M. F. Cassani, M. S. Lucero, V. Parreño, S. C. Zoth, and A. Berinstein. 2020. Development of diagnostic tools for IBDV detection using plants as bioreactors. AMB Express 10:95.

Heine, H. G., M. Haritou, P. Failla, K. Fahey, and A. Azad. 1991. Sequence analysis and expression of the host-protective immunogen VP2 of a variant strain of infectious bursal disease virus which can circumvent vaccination with standard type I strains. J. Gen. Virol. 72:1835–1843.

Hernández, M., G. Tomás, A. Marandino, G. Iraola, L. Maya, N. Mattion, D. Hernández, P. Villegas, A. Banda, Y. Panzera, and R. Pérez. 2015. Genetic characterization of South American infectious bursal disease virus reveals the existence of a distinct worldwide-spread genetic lineage. Avian Pathol. 44:212–221.

Jackwood, D. H., and Y. M. Saif. 1987. Antigenic diversity of infectious bursal disease viruses. Avian Dis. 31:766–770.

Jaton, J., M. S. Lucero, M. Richetta, S. Pinto, M. J. Gravisaco, A. Berinstein, E. Gómez, and S. Chimeno Zoth. 2022. Comparative study of the immune response induced by an Argentinian classical strain of IBDV in two chicken breeds. Vet. Med. Int. 2022:6255367 eCollection2022.

Jeon, W.-J., K.-S. Choi, D.-W. Lee, E.-K. Lee, S.-H. Cha, S.-H. Cho, J. H. Kwon, Y. S. Yoon, J. H. Kim, and H.-J. Kwon. 2009. Molecular epizootiology of infectious bursal disease (IBD) in Korea. Virus Genes 39:342–351.

Kim, I. J., M. Gagic, and J. M. Sharma. 1999. Recovery of antibody-producing ability and lymphocyte repopulation of bursal follicles in chickens exposed to infectious bursal disease virus. Avian Dis. 43:401–413.

Ley, D. H., R. Yamamoto, and A. A. Bickford. 1983. The pathogenesis of infectious bursal disease: serologic, histopathologic, and clinical chemical observations. Avian Dis. 27:1060–1085.

Lisowska, A., A. Pikuła, J. Opolska, A. Jasik, A. Kycko, and K. Domańska-Blicharz. 2021. Virulence properties of GI-23 infectious bronchitis virus isolated in Poland and efficacy of different vaccination strategies. Pathogens 10:522.

Lupini, C., G. Quaglia, G. Mescolini, E. Russo, R. Salaroli, M. Forni, S. Boldini, and E. Catelli. 2020. Alteration of immunological parameters in infectious bronchitis vaccinated-specific pathogen-free broilers after the use of different infectious bursal disease vaccines. Poult. Sci. 99:4351–4359.

Marandino, A., and R. Pérez. 2021. Genetic and antigenic diversity of infectious bronchitis virus in South America. Avian Dis. 65:624–630.

Marandino, A., G. Tomás, Y. Panzera, G. Greif, A. Parodi-Talice, M. Hernández, C. Techera, D. Hernández, and R. Pérez. 2017. Whole-genome characterization of Uruguayan strains of avian infectious bronchitis virus reveals extensive recombination between the two major South American lineages. Infect. Genet. Evol. 54:245–250.

Marandino, A., A. Vagnozzi, M. I. Craig, G. Tomás, C. Techera, Y. Panzera, F. Vera, and R. Pérez. 2019. Genetic and antigenic heterogeneity of infectious bronchitis virus in South America: implications for control programmes. Avian Pathol. 48:270–277.

Marandino, A., A. Vagnozzi, G. Tomás, C. Techera, R. Gerez, M. Hernández, J. Williman, M. Realpe, G. Greif, Y. Panzera, and R. Pérez. 2022. Origin of new lineages by recombination and mutation in avian infectious bronchitis virus from South America. Viruses 14:2095.

Michel, L. O., and D. J. Jackwood. 2017. Classification of infectious bursal disease virus into genogroups. Arch. Virol. 162:3661–3670.

Najimudeen, S. M., C. Barboza-Solis, A. Ali, S. M. Buharideen, I. M. Isham, M. S. H. Hassan, D. Ojkic, G. Van Marle, S. C. Cork, F. van der Meer, M. Boulianne, and M. F. Abdul-Careem. 2022. Pathogenesis and host responses in lungs and kidneys following Canadian 4/91 infectious bronchitis virus (IBV) infection in chickens. Virology 566:75–88.

OIE. 2017. Chapter 2.3.2. Avian infectious bronchitis (NB: Version adopted in May 2013). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Paris, France.

Ojkic, D., E. Martin, J. Swinton, B. Binnington, and M. Brash. 2007. Genotyping of Canadian field strains of infectious bursal disease virus. Avian Pathol. 36:427–433.

Pejkovski, C., F. G. Davelaar, and B. Kouwenhoven. 1979. Immunosuppressive effect of infectious bursal disease virus on vaccination against infectious bronchitis. Avian Pathol. 8:95–106.

Raji, A., S. Oladele, and A. Jibril. 2017. Bursa body index as a visual indicator for the assessment of bursa of Fabricius. J. Vet. Med. Anim. Health 9:32–38.

Rimondi, A., M. I. Craig, A. Vagnozzi, G. König, M. Delamer, and A. Pereda. 2009. Molecular characterization of avian infectious bronchitis virus strains from outbreaks in Argentina (2001-2008). Avian Pathol. 38:149–153.

Saif, Y. M. 1991. Immunosuppression induced by infectious bursal disease virus. Vet. Immunol. Immunopathol. 30:45–50.

Sapats, S. I., and J. Ignjatovic. 2000. Antigenic and sequence heterogeneity of infectious bursal disease virus strains isolated in Australia. Arch. Virol. 145:773–785.

Sharma, J. M., I. J. Kim, S. Rautenschlein, and H. Y. Yeh. 2000. Infectious bursal disease virus of chickens: pathogenesis and immunosuppression. Dev. Comp. Immunol. 24:223–235.

Singh, J., H. S. Banga, R. S. Brar, N. D. Singh, S. Sodhi, and G. D. Leishangthem. 2015. Histopathological and immunohistochemical diagnosis of infectious bursal disease in poultry birds. Vet. World 8:1331–1339.

Skeeles, J. K., P. D. Lukert, E. V. De Buysscher, O. J. Fletcher, and J. Brown. 1979. Infectious bursal disease viral infections. II. The relationship of age, complement levels, virus-neutralizing antibody, clotting, and lesions. Avian Dis. 23:107–117.

Snyder, D. B., D. P. Lana, P. K. Savage, F. S. Yancey, S. A. Mengel, and W. W. Marquardt. 1988. Differentiation of infectious bursal disease viruses directly from infected tissues with neutralizing monoclonal antibodies: evidence of a major antigenic shift in recent field isolates. Avian Dis. 32:535–539.

Tomás, G., A. Marandino, C. Courtillon, M. Amelot, A. Keita, A. Pikula, M. Hernández, D. Hernández, A. Vagnozzi, Y. Panzera, K. Domańska-Blicharz, N. Eterradossi, R. Pérez, and S. M. Soubies. 2019. Antigenicity, pathogenicity and immunosuppressive effect caused by a South American isolate of infectious bursal disease virus belonging to the "distinct" genetic lineage. Avian Pathol. 48:245–254.

Valastro, V., E. C. Holmes, P. Britton, A. Fusaro, M. W. Jackwood, G. Cattoli, and I. Monne. 2016. S1 gene-based phylogeny of infectious bronchitis virus: an attempt to harmonize virus classification. Infect. Genet. Evol. 39:349–364.

van den Berg, T. P., N. Eterradossi, D. Toquin, and G. Meulemans. 2000. Infectious bursal disease (Gumboro disease). Rev. Sci. Technol. 19:509–543.

Vera, F., M. I. Craig, V. Olivera, F. Rojas, G. König, A. Pereda, and A. Vagnozzi. 2015. Molecular characterization of infectious bursal disease virus (IBDV) isolated in Argentina indicates a regional lineage. Arch. Virol. 160:1909–1921.

Xu, A.-H., L. Sun, K.-H. Tu, Q.-Y. Teng, J. Xue, and G.-Z. Zhang. 2021. Experimental co-infection of variant infectious bursal disease virus and fowl adenovirus serotype 4 increases mortality and reduces immune response in chickens. Vet. Res. 52:61.

Yamazaki, K., H. Ohta, T. Kawai, T. Yamaguchi, T. Obi, and K. Takase. 2017. Characterization of variant infectious bursal disease virus from a broiler farm in Japan using immunized sentinel chickens. J. Vet. Med. Sci. 79:175–183.