Histopathological findings in egg-laying hens infected with avian infectious bronchitis virus

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ABSTRACT

In order to dissect the histopathological changes produced by the infection of avian infectious bronchitis virus in previously vaccinated egg-laying hens from a poultry farming unit, 35 White Leghorn egg-laying hens that had been in production for 9 to 10 months (twenty seven of which had clinical symptoms corresponding to respiratory disease and eight apparently healthy individuals) were selected for further study. After clinical examination and necropsy, they were classified into apparently healthy, mild, moderate or severe according to the severity of the clinical-pathological process. Samples were taken from paranasal sinuses, trachea and lungs for histopathological study, and trachea-lung pools were prepared from four individuals for virus isolation and molecular biology assays. The presence of mucus was evidenced with Schiff's non-enzymatic histochemical staining, and histomorphometric analyses were used to estimate the number of glands in the tracheal mucosa. The proportions of histopathological lesions were compared, using one-way Anova to determine gland loss at the tracheae with a significance level of $p < 0.05$ in both cases. Histopathological analysis of the epithelia of paranasal sinuses, trachea and bronchia revealed the presence of epithelial erosion, mucous exudate and hyperplasia of mucosa-associated lymphoid tissue. Glandular cysts were observed at the paranasal sinuses, and epithelial metaplasia was detected in the trachea. It was possible to isolate and identify infectious bronchitis coronavirus from the original samples and from samples passaged in chicken embryos.

Keywords: glandular atrophy, avian infectious bronchitis, epithelial metaplasia, bronchus-associated lymphoid tissue

RESUMEN

Hallazgos histopatológicos en gallinas ponedoras afectadas por el virus de la bronquitis infecciosa aviar. Con el objetivo de determinar los cambios histopatológicos causados por el virus de bronquitis infecciosa aviar en gallinas ponedoras vacunadas, afectadas con síndrome respiratorio crónico, en una unidad avícola, se seleccionaron 35 gallinas White Leghorn, entre 9 y 10 meses de postura (27 con diagnóstico clínico presuntivo de enfermedad respiratoria y ocho sin alteraciones aparentes. Se les realizó el examen clínico y la necropsia, y se clasificaron en cuatro grupos según el proceso clínico-patológico: leve, moderado, severo y control sano. Se tomaron muestras de los senos paranasales, la tráquea y los pulmones (histopatología) y mezclas de muestras de tráquea-pulmón, de tres y cuatro aves para el aislamiento del coronavirus de la bronquitis infecciosa y el análisis de biología molecular, respectivamente. Se aplicó la técnica histoquímica no enzimática del ácido periódico de Schiff, para demostrar la presencia de moco; y se cuantificaron las glándulas de la mucosa de la tráquea (histomorfometría). Se compararon las proporciones de las lesiones histopatológicas y se hizo un análisis de varianza de una vía para determinar las pérdidas de las glándulas en la tráquea, con un nivel de significación en ambos análisis de $p < 0.05$. Mediante técnicas histopatológicas, en el epitelio de los senos paranasales, la tráquea y los bronquios, se observó erosión del epitelio y exudado mucoso e hiperplasia del tejido linfoide asociado a mucosa. En los senos paranasales se observaron quistes glandulares y en la tráquea se observó metaplasia epitelial. A partir de muestras originales y pases en embrión de pollos se aisló e identificó el coronavirus de la bronquitis infecciosa.

Palabras clave: atrofia glandular, bronquitis infecciosa aviaria, metaplasia epitelial, tejido linfoide asociado a bronquio

Introduction

Although egg-laying hens are a highly specialized hybrid species, realizing their full genetic and productive potential requires strict handling practices and almost extreme biosafety protocols and procedures that must be implemented along every stage of the production process. This situation is a consequence of, among other factors, the high potential for outbreaks of acute or chronic respiratory disease that characterizes intensive farming settings, with causative agents ranging from bacteria or avian mycoplasmas to pathogenic fungi or viruses [1, 2]. One prominent example of the latter case is that of avian infectious bronchitis virus (IBV), a gammacoronavirus belonging to the *Coronaviridae* family, in the order *Nidovirales* [3].

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IBV is a highly infectious virus with a geographic distribution spanning the entire world. Infections with this virus exact a heavy economic toll on the poultry industry, as they produce severe weight loss in layer flocks and decrease egg production and quality, ultimately raising rejection rates at downstream processing plants [4, 5]. Morbidity due to this cause is generally high (from 90 to 100%), but mortality is low (5%), although the latter increases in the case of nephropathogenic strains [6, 7].

The main clinical symptoms exhibited by laying hens affected by this virus include serous conjunctivitis, dyspnea and, ultimately, asphyxia. Its most important consequence is the reduction of egg production rates, which may reach 40% in chronic cases. Although productivity usually rebounds after 4 to 5 weeks, previous production levels are seldom recovered. The affected eggs are usually deformed, whitish, porous, exhibiting calcareous excrescences or even lacking the shell in rare cases. Their albumen is orangey amber, and there is no distinction between aqueous and dense zones. During mild respiratory infections it is common to detect renal alterations such as inflammation and discoloration of kidneys, presence of urate salts at the ureters (urolithiasis) and visceral gout. Nephropathogenic IBV pathotypes can cause this symptomatology [8].

Among the anatomopathological characteristics of mild cases of respiratory disease are excessive mucus, which can even become sebaceous –especially in broilers– and pulmonary congestion and opacity, with engrossed air sac walls. In severe cases there is also abundant mucus, producing severe inflammation with reddening of the tracheal rings in older chicken and asphyxia in younger individuals [9]. Histologically, there is epithelial hyperplasia and metaplasia, as well as loss of cilia, in both trachea and bronchi, and superficial cells are often engrossed. Subepithelial engrossment zones are characterized by edema and infiltration of the lamina, mainly by monocytes and lymphocytes [10].

The laboratory diagnosis of IBV requires isolating or directly detecting the virus, although serological techniques can be useful under some circumstances. Serotyping is done using hemagglutination inhibition assays, employing ELISA instead for serological diagnosis. Other techniques used for this purpose have included electron microscopy [9], assays based on monoclonal antibodies [11], viral neutralization assays [12] and, more recently, tests based on reverse transcriptase-polymerase chain reaction (RT-PCR) combined with restriction fragment length polymorphism to identify viral genotypes [13-15].

The continuous appearance and emergence of new serotypes has complicated viral diagnosis and the design of effective control and management programs, as the resulting antigenic variation decreases the cross-protection afforded by vaccine strains against field strains of distantly related genotypes or serotypes [16].

Control of IBV in many countries is achieved mainly through a combination of biosafety procedures and live or inactivated vaccines conferring a specific immune response [17]. In Cuba there are immunization programs against avian infectious bronchitis based

on the application of live and inactivated vaccines in breeder and layer flocks, respectively [18, 19]. Despite the implementation of control procedures and biosafety practices, however, outbreaks of respiratory syndrome with high morbidity and low mortality have continued to affect intensive poultry farming facilities.

For the above reasons, it was decided to examine the histopathological changes caused by infections of the avian bronchitis virus in vaccinated egg-laying hens affected by chronic respiratory syndrome.

*M*aterials and methods

Bird selection

Thirty-five White Leghorn egg-laying hens approximately 38 weeks old were randomly selected from a poultry farming unit (twenty seven with a clinical diagnosis of respiratory disease, and eight apparently healthy birds that were used as a control group).

They were fed a balanced diet, and their handling complied with current technical guidelines and regulations of the country, in force since the decade of the 1980s [20].

Immunization schedule

The birds received three doses of live vaccine (strain H120, Massachusetts serotype) at 1, 35 and 85 days of life, following the immunization program currently used in the country [20].

Clinical examination and sacrifice of the birds

Both the clinical examination and sacrifice of the birds followed the methodology described by Sánchez [21]. Gross examinations were performed during necropsy, scoring the severity of clinical manifestations and recording existing anatomopathological lesions. The birds were then classified into four groups (apparently healthy, mild, moderate and severe) according to the severity of the clinical-anatomopathological alterations noticeable during gross examinations (see Table).

Sampling and sample processing

Organs for the histopathological study

Sample fragments of paranasal sinuses, trachea and lung were extracted and stored in 4% formaldehyde in saline phosphate buffer. The paranasal sinuses samples were softened by placing them for 21 days in a decalcification solution.

Organs for the virological and molecular study

Trachea and lung fragments from three and four birds were taken and randomly pooled, per organ, in two groups of four and one of three (from 11 birds in total). The pools were stored at -20 °C in sterile 50 mL plastic tubes without culture medium until used.

Histopathological technique

Fragments of paranasal sinus, trachea and lungs stored in a solution of formaldehyde with saline phosphate buffer at 4% were processed by inclusion into paraffin, sectioning and staining with hematoxylin-eosin. In addition, they were also processed with Schiff's

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Table. Classification of the birds into groups according to the severity of respiratory symptoms (n=35)

Groups	Proportion	Clinical signs	Macroscopic lesions	Microscopic lesions
Mild	8	Serous nasal secretion, conjunctivitis, dyspnea, sneezing, retraction of comb and wattles, mild weight loss	Serous rhinitis, genital atrophy, ovarian degeneration	Discrete MALT* hyperplasia in paranasal sinuses and trachea Partial erosion of respiratory epithelium with loss of cilia in the trachea Discrete glandular degeneration of glandular epithelium in the paranasal sinuses
Moderate	8	Facial tumefaction, tearing, conjunctivitis, sero-catarrhal fibrinoid nasal secretion, sneezing, dyspnea, worn-out feathers, retraction of comb and wattles, weight loss	Sero-catarrhal rhinitis, fibrinous rhinitis, splenomegaly, necrotic foci at the liver, ovarian degeneration with deformed follicles	MALT hyperplasia, moderate erosion and damage to glands of the respiratory epithelium, paranasal sinuses and trachea Muco-fibrinous exudate in paranasal sinuses
Severe	11	Facial tumefaction with hard consistency and loss of vision, tearing, conjunctivitis, dyspnea, catarrhal nasal secretion	Fibrinous-catarrhal rhino-conjunctivitis, focal pneumonia, liver degeneration and congestion	MALT hyperplasia and atrophy of glands of the respiratory epithelium of paranasal sinuses, trachea and bronchi Cystic glandular degeneration Metaplasia of respiratory epithelium Bronchitis and catarrhal bronchiolitis
Healthy control	8	No clinical signs	No alterations	No alterations

*MALT: Mucosa-associated lymphoid tissue.

non-enzymatic periodic acid histochemical staining (PAS), as described by Vacca [22].

Quantification of epithelial glands at the trachea of animals with respiratory processes of varying severity

This technique employed 35 tracheal rings from all 35 birds used in the study. Gland atrophy was determined by histomorphometry of one ring from each trachea from animals falling into different levels of the chronic respiratory syndrome classification scale, based on the macroscopic clinical-anatomopathological characteristics described above.

Viral isolation and molecular identification

Samples of trachea and lungs from 11 hens in groups of three and four birds were taken after necropsy. These samples were processed and stored at -80 °C until inoculated into chicken embryos.

Organ homogenates were inoculated into 9 to 11 day-old chicken embryos, injecting 0.25 mL of the sample into the allantoic cavity and using 10 embryos for each sample. The embryos were incubated at 37 °C, and their viability was checked daily. At 72 h after inoculation, the allantoic fluid was collected, performing two to three blind passages in chicken embryos.

Allantoic fluid collected from each passage was evaluated using an RT-PCR assay for IBV.

Vaccine strain H120, used in the immunization program currently implemented in Cuba [20], was used as positive control.

RNA extraction

RNA was extracted using TRI Reagent® LS, following the instructions from the manufacturer (Sigma, USA). The obtained RNA was resuspended in 10 µL of nuclease-free water (Promega, USA).

RT-PCR

Viral RNA in the obtained RNA samples was detected with a semi-nested RT-PCR, using primers: sense UTR 41 - 5´ATG TCT ATC GCC AGG GAA ATG TC 3´); antisense UTR 11 - 5´ GCT CTA ACT CTA TAC TAG CCT A 3´ and UTR 31- 5´ GGG CGT CCA AGT GCT GTA CCC 3´. These primers bind to a region of the 3´ untranslated region (UTR) that is highly conserved across IBV genotypes [23].

Statistical analysis

The proportions of the principal histopathological lesions were compared, and a one-way analysis of variance (Anova) was performed to evaluate the loss of epithelial glands at the trachea, as implemented in the statistical software packages Comprop-1 and Statgraphics Plus 5.1 (Statistical Graphics Corporation, USA). A statistical significance level of $p < 0.05$ was chosen for both analyses.

*R*esults

The main histological changes in paranasal sinuses, trachea and lungs of laying hens, grouped according to their score in classification scheme used during gross examinations, are shown in the table.

The respiratory epithelium was markedly eroded, and there was degeneration of acinotubular glands. In individuals classified as severe there were glandular cysts with mucous exudation (Figure 1).

Another important histopathological finding is the presence of hyperplastic acinotubular glands. The latter are totally full of mucus, and secrete their contents

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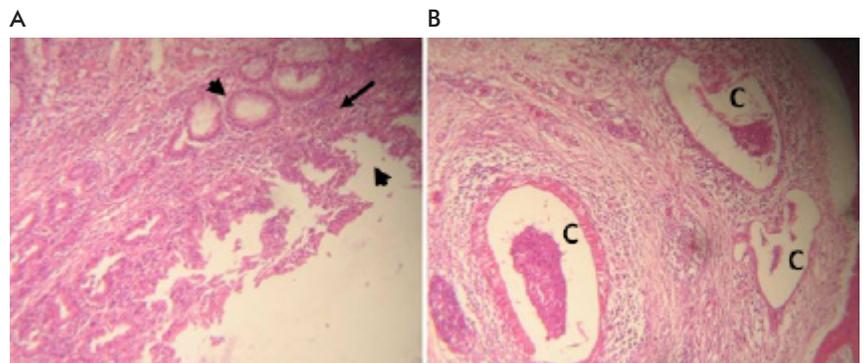
Figure 1. Microphotographs of paranasal sinuses (respiratory region; 400 ×) stained with hematoxylin-eosin. A) Hyperplasia of nasal-associated lymphoid tissue (arrow) and marked erosion of the respiratory epithelium with degeneration of acinotubular glands (arrowheads). B) Glandular cysts (C).

into the lumen with some degree of distension (Figures 2A and B), as described in the table.

At the trachea there was moderate loss of cilia and hyperplasia of the bronchus-associated lymphoid tissue (BALT). In advanced stages of the respiratory infection there was also metaplasia of the cylindrical pseudostratified epithelium to flat cells, with submucosal engrossment (Figures 2C and D).

Histopathological analysis of the respiratory system also revealed changes in bronchi, such as BALT hyperplasia and a catarrhal exudative inflammatory response both in epithelial glands and the bronchial lumen (Figures 2E and F).

Upon analysis of PAS-stained sections of the respiratory epithelium of paranasal sinuses, trachea and bronchi, it was possible to confirm the presence of catarrhal exudates (mucous; figure 3).

There were statistically significant differences regarding the degree of epithelial gland loss in the tracheal rings of groups exhibiting the clinical-pathological alterations of chronic respiratory disease when compared to the control group, which had no apparent alterations. The third group (severe) exhibited the highest pathological significance (Figure 4).

The viral isolation assays employing three successive passes in chicken embryos produced symptoms not unlike those of IBV when infecting adult individuals: mortality, stunted growth and hemorrhagic embryos. Successful viral isolation was confirmed by RT-PCR analysis of clinical samples, which produced amplicons whose relative electrophoretic mobility (179 bp) matched that expected for the employed primers (Figure 5).

*D*iscussion

Avian bronchitis virus infection starts in the upper respiratory system, where it induces the secretion of mucus by goblet cells at the mucosal epithelium [24]. Most strains of this virus are able to replicate in the upper respiratory tract without producing apparent clinical signs. When clinical signs are present, the progression of lesions in this system is divided in three stages: degenerative, hyperplastic and regenerative [25]. The severity of histopathological findings paralleled the scale based on clinical signs used to classify the groups with respiratory infection (mild, moderate and severe). Some of the most conspicuous findings include the erosion of the epithelium with degenerative damage of paranasal sinus glands, BALT hyperplasia, and glandular hyperplasia with mucus hypersecretion throughout the respiratory epithelium with loss of cilia (paranasal sinuses, trachea and primary bronchi). The latter microscopic alterations, specifically those in the trachea, are defense mechanisms due to ciliary movement and the exudation of mucus by goblet cells during IBV infection [26]. These alterations, which characterize the acute stage of the disease, can be easily observed in the trachea by electron microscopy due to the anatomical simplicity of this organ [9].

Virulent strains of IBV produce epithelial damage, loss of cilia and hyperplasia. These effects predispose the individual to coinfections with opportunistic pathogens, such as *Escherichia coli* [27]. This enterobacterium often aggravates respiratory disease, leading in
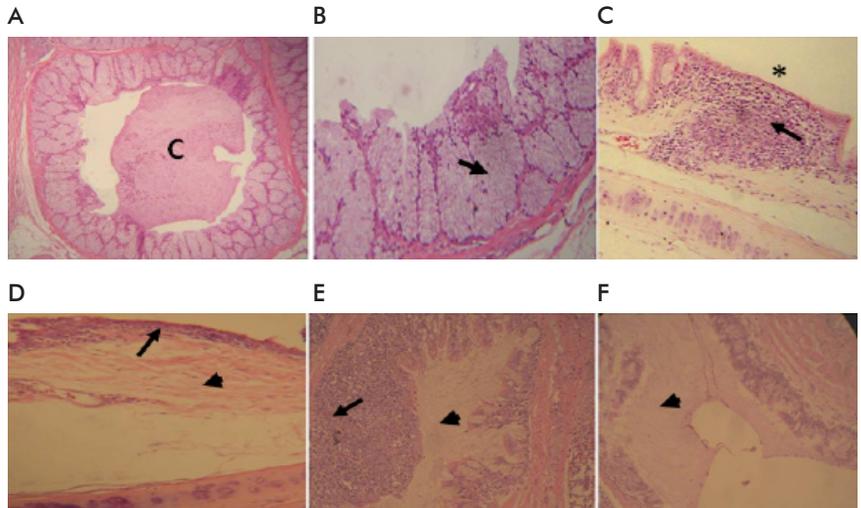
Figure 2. Microphotograph of paranasal sinuses stained with hematoxylin-eosin (respiratory region, 400 ×). A) Fibrinous exudate clot (C). B) Hyperplasia of acinotubular glands (arrow). C) Loss of cilia (asterisk) and hyperplasia of trachea-associated lymphoid tissue (arrow). D) Metaplasia of respiratory epithelium (arrow) with submucosal engrossment (arrowhead). E) Hyperplasia of trachea-associated lymphoid tissue (arrow) and mucus hypersecretion (arrowhead) in bronchi F). Hypersecretion of mucus (arrowhead).
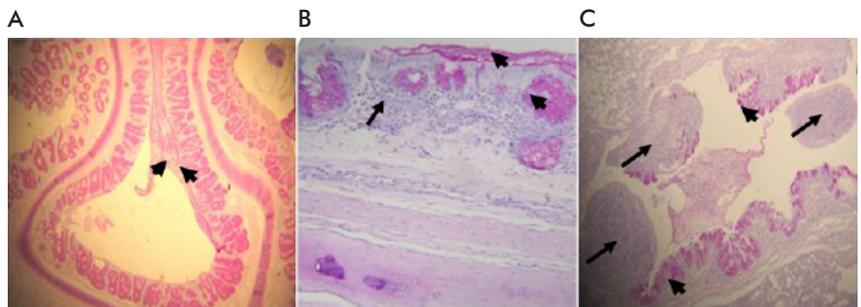
Figure 3. Microphotograph of the respiratory organ (200 ×). Specifically of paranasal sinuses (A), trachea (B) and tertiary bronchus (C) using Schiff's non-enzymatic periodic acid staining. Hypersecretion of mucus in epithelium and submucosal glands (arrowhead) and hyperplasia in trachea-associated lymphoid tissue (arrow) are indicated.
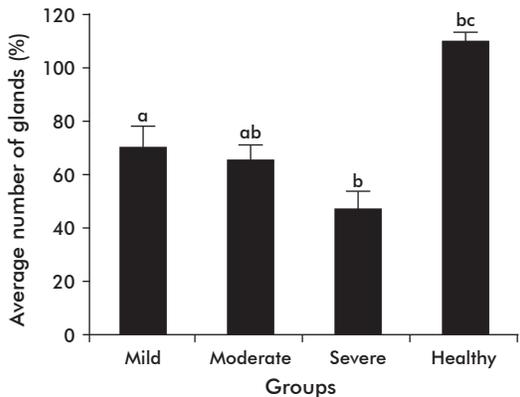
Figure 4. Quantification of the average number of glands in the epithelium of one tracheal ring in individuals undergoing respiratory disease at varying levels of severity. The data have been transformed to frequency ± SD (standard deviation). Different letters denote statistical significant differences (one-way Anova, $p < 0.05$).
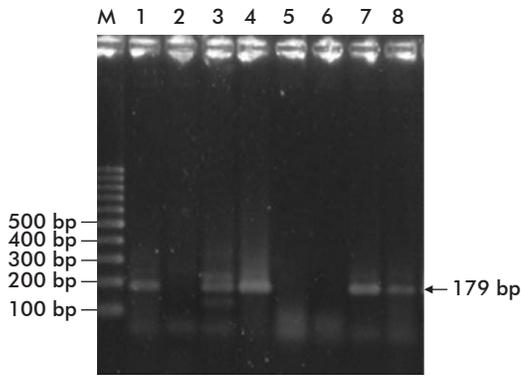
Figure 5. Agarose gel electrophoresis (2%) of RT-PCR reactions to detect avian infectious bronchitis virus in suspect field samples. M: 100 bp molecular weight ladder (Promega); Lane 1: positive control (strain M41); 2: negative control (nuclease-free water); 3: original samples from animals 13 to 15; 4: second passage in chicken embryos of samples from animals 13 to 15; 5: original samples of animals 9 to 12; 6: third passage in chicken embryos of samples from animals 9 to 12; 7: third passage in chicken embryos of samples from animals 16 to 19; 8: fourth passage in chicken embryos of samples from animals 16 to 19.

many cases to the death of infected individuals [28].

Another important histopathological finding is the presence of epithelial metaplasia, with characteristics resembling those of flat cells, and the engrossment of tracheal submucosa. These results coincide with those of an earlier study published in 2003 [29].

Controlling IBV infections through vaccination is difficult and not always successful, due to the continuous emergence of new viral serotypes and variants exhibiting very low levels of cross-protection [30, 31]. The origin of this antigenic variation is multifactorial, and includes among its causes the selective immunological pressure exerted by the widespread application of vaccines, the high frequency of coinfections -leading to recombination events as an additional source of variation- and the disappearance of once dominant serotypes due to vaccination, followed by their replacement by different field strains [32, 33].

Many different IBV vaccines -mostly against variants of the Massachusetts strain- have been developed internationally, and their efficacy in broilers and laying hens has been well studied. Most of them, however, are prone to causing the disease themselves, and the protection they provide is poor or nil [34], as reported in 1992 for the DE 072 [35, 36] and GA98 [37, 38] variants in the USA. A single group of IBV has been described in Brazil, subdivided in three subgroups together with genotype 4/91 [39-41]. Variants of the Massachusetts strains were also reported in Chile during the 1980s [42], while the Dutch serotypes (D207, D212, D3896 and D3128) have been described in Europe [43]. An IBV strain was isolated in 1980 in Africa and found to be responsible for severe respiratory problems [39, 44]. Additional Massachusetts strain variants were serotyped for Israel, during the mid 1990s [13, 45, 46] and other IBV variants were described in Korea during the mid 1980s [47]. Although a Massachusetts strain-based vaccine was used with good results in the latter case, its success was short-lived, as outbreaks of infectious bronchitis, with a high incidence of renal complications, have been taking place since 1990 in vaccinated flocks from Korea. One possible cause was uncovered by Lee *et al.*, who found a high degree of genetic diversity among the IBV variants isolated from the diseased animals [48]. Some of these variants are indigenous, while others are genetically related to IBV variants in neighboring countries [47], suggesting that IBV strains in Korea are evolving continuously [49]. In the case of Cuba, the results suggest that other, yet to be studied variants or serotypes of the Massachusetts strain may be currently circulating.

Risk of HBV infection is also influenced by other factors, such as complete or partial vaccine coverage failures, lower vaccine efficacy against heterologous strains, presence of immunosuppressive agents, inadequate immunization schedules, improper immunization technique, variations in immunization technique (for instance, in the amount, quality and temperature of the water used to dilute the vaccine, or in the inoculated dose); and the use of vaccine combinations against different agents [50, 51].

In this work, it was possible to isolate and identify the IBV in hens, starting from the evaluation of histopathological findings in the respiratory system. Some authors state that it is not always possible to identify IBV in flocks for several reasons. One possible cause is the appearance of viral mutations, due not only to point nucleotide changes or insertions/deletions, but to recombination events between unrelated strains, whose frequency is significant due to the common occurrence of IBV coinfections in chicken [23, 52]. Although such hybrid or chimeric viruses will sometimes replicate better, the existence of differences in genetic regions is highly probable [53]. Another possible cause is the movement of flocks and the mixing of layer hen batches, which together propitiate coinfections by unrelated strains and thus, the occurrence of recombination events between separate IBV lineages.

Summarizing, the present work described the histopathological changes in the epithelium of paranasal sinuses, trachea and bronchi of egg-laying hens affected by the infectious bronchitis coronavirus, as confirmed by viral isolation in chicken embryos and identification by RT-PCR.

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