Assessment of molecular and genetic evolution, antigenicity and virulence properties during the persistence of the infectious bronchitis virus in broiler breeders

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Abstract

The infectious bronchitis virus (IBV) causes a highly contagious disease [infectious bronchitis (IB)] that results in substantial economic losses to the poultry industry worldwide. We conducted a molecular and phylogenetic analysis of the S1 gene of Brazilian (BR) IBV isolates from a routinely vaccinated commercial flock of broiler breeders, obtained from clinical IB episodes that occurred in 24-, 46- and 62-week-old chickens. We also characterized the antigenicity, pathogenesis, tissue tropism and spreading of three IBV isolates by experimental infection of specific pathogen-free (SPF) chickens and contact sentinel birds. The results reveal that the three IBV isolates mainly exhibited mutations in the hypervariable regions (HVRs) of the S1 gene and protein, but were phylogenetically and serologically closely related, belonging to lineage 11 of the GI genotype, the former BR genotype I. All three isolates caused persistent infection in broiler breeders reared in the field, despite high systemic anti-IBV antibody titres, and exhibited tropism and pathogenicity for the trachea and kidney after experimental infection in SPF chickens and contact birds. In conclusion, BR genotype I isolates of IBV evolve continuously during the productive cycle of persistently infected broiler breeders, causing outbreaks that are not impaired by the current vaccination programme with Massachusetts vaccine strains. In addition, the genetic alterations in the S1 gene of these isolates were not able to change their tissue tropism and pathogenicity, but did seem to negatively influence the effectiveness of the host immune responses against these viruses, and favour viral persistence.

INTRODUCTION

Coronaviruses (CoVs) are found in a wide variety of animals, including humans, in which they can cause respiratory, enteric, hepatic and neurological diseases of varying severity. The *Coronaviridae* are classified in the order *Nidovirales* and are divided into two subfamilies, *Coronavirinae* and *Torovirinae*. The coronavirinae are further subdivided into the genera *Alphacoronavirus*, *Betacoronavirus*, *Deltacoronavirus* and *Gammacoronavirus*.

The infectious bronchitis virus (IBV) belongs to the *Gammacoronavirus* genus and causes a highly contagious upper respiratory disease, although some strains of the virus can affect the uro-genital tract and cause interstitial nephritis and reproductive disorders [1–4]. The disease can induce

severe economic losses, especially with relation to decreases in egg production, poor eggshell quality, reduced hatchability [5], increased feed conversion and carcass condemnation at slaughterhouse [6].

The S1 subunit of the envelope spike glycoprotein of IBV contains the receptor-binding domain, is a determinant of cell tropism [7] and carries virus-neutralizing and serotype-specific determinants of this virus. The high mutation rate of the S1 gene during IBV replication generates extensive antigenic variability in the progeny of this virus, hampering the control of IBV infection by vaccination. This implies the existence of multiple serotypes and variants of the virus that the current IBV vaccine strains may not offer full cross-protection against [1, 8, 9]. The S1 subunit protein is the major immunogen of IBV, which contains epitopes that can induce the production

of specific neutralizing antibodies and the haemagglutination inhibition antibody [10–12]. Different serotypes of IBV isolates usually exhibit poor cross-protection [13–15].

Since the 1990s, and after the characterization of different serotypes circulating in Brazilian flocks [16], an indigenous IBV genotype (BR genotype) and serotype became predominant in Brazil [17–19].

More recently, phylodynamic analysis demonstrated that Brazilian genotype IBV strains, and other isolates from Uruguay and Argentina, comprise an exclusive lineage that emerged in the 1960s and is genetically and antigenically distinct from the Massachusetts (Mass) vaccine strains. Thus, it is expected that low vaccine protection will be conferred by the Mass genotype against these variant IBV strains, because of the genetic differences and phylogenetic distance between the South American and Mass genotype IBV strains. This may explain the successful spreading of these IBV variants in most South American countries, despite the continuous use of Mass vaccines to control IBV infection [20].

The continuous emergence of new genotypes and serotypes of IBV is due to the accumulation of mutations, especially in the S1 gene, over time, as well as recombination events. In addition, there are different determinants in the microenvironment inside the host, including immune responses followed by selection pressures, as well as affinity to different cell receptors and physico-chemical conditions involved in the selection process [21, 22]. Thus, IBV subpopulations differing in S1 gene sequences are present in commercial attenuated vaccine preparations and are usually positively selected for during passage in the chicken host. These positively selected IBV subpopulations can contribute to the selection of a more adapted IBV strain that has an increased ability to evade host immune responses [23, 24] or infect cells or tissues located in immune-privileged sites, and so can establish persistent infections in birds [25].

New IBV genotypes and serotypes are therefore continually emerging, but little is known about the evolution of these new variants in birds reared in poultry production systems or the most relevant biological changes affecting them, and in particular the data on the characterization of IBV strains isolated from chickens of long productive cycles, such as breeders, are lacking. In this study we analysed three IBV isolates obtained from three clinical IB episodes affecting persistent infected breeders molecularly and phylogenetically. We also characterized the serotype, pathogenicity, tissue tropism and spreading ability of these IBV isolates, and looked for possible associations between their S1 gene sequences and some of their phenotypic properties.

RESULTS

Clinical–pathological assessment in broiler breeders

Only one of the three broiler breeder farms studied had viral persistence and Mass and non-Mass IBV strains detected in samples from different organs during all productive cycles (24, 28, 32, 35, 43, 46, 50, 55, 58, 62 and 65 weeks of age). The samples that were detected as 'non–Mass'-only were submitted to isolation in embryonating specific pathogen-free (SPF) chicken eggs. IBV isolates were obtained from samples collected at 24, 46, and 62 weeks of age. Coincidentally, the major clinical manifestations occurred at 24, 46 and 62 weeks of age, and the birds showed depression, with ruffled feathers and fever. The affected flocks had 851/14.826 (5.74 %), 101/13.467 (0.75 %) and 62/12.400 (0.50 %) mortality rates at 24, 46 and 62 weeks of age, respectively. Histopathological analysis from trachea showed mild to moderate degeneration and necrosis of the ciliated epithelial cells. Mild to moderate renal lesions, including degeneration, necrosis of renal tubular epithelial cells and lymphocytic infiltration in the interstitium were only observed in samples collected at 24 weeks of age.

All breeders were positive for anti-IBV antibodies as determined by ELISA (Fig. 1a). The red dots in Fig. 1a show that the serum samples collected at 28, 50 and 65 weeks of age showed increased antibody titres against IBV, and these time-points corresponded to the serum samples collected immediately after the periods of infectious bronchitis clinical manifestations, or increased viral loads at 24, 46 and 62 weeks of age, respectively.

The mean of the viral load from five broiler breeders showed that relevant high viral RNA loads were detected in respiratory and non-respiratory tissues, reaching 4.88 $\log_{10}$ in the trachea, 4.2 $\log_{10}$ in the kidney and 5.27 $\log_{10}$ in the cecal tonsils (Fig. 1b). Overall, the viral loads were lower in kidney samples than in trachea and cecal tonsil samples, except at the age of 43 and 55 weeks, when 3.8 and 2.38 $\log_{10}$ RNA copies were detected in the kidneys, respectively, while there was no detection of RNA copies in the trachea in both intervals.

Phylogenetic analysis and comparisons of S1 gene sequences

A phylogenetic tree was generated to describe the relationship between the nucleotide sequences of the full S1 gene of Brazilian IBV isolates from 24- (IBV/24W, GenBank KY565553), 46- (IBV/46W, GenBank KY565555) and 62-week-old chickens (IBV/62W, GenBank KY565554), including other field strains from Brazil and reference strains from Genbank. The phylogenetic tree showed that the IBV/24W, IBV/46W and IBV/62W strains isolated in this study belonged to lineage 11 of genotype I, and comprised viruses isolated between 1975 and 2013 from the states of Minas Gerais and São Paulo in Brazil (Fig. 2). For the S1 gene, the identity ranged from 85.3 to 93.1 % between IBV isolates in this study and other Brazilian IBV variant strains. In contrast, the S1 gene nucleotide sequences of three IBV Brazilian isolates showed lower identity with the vaccine IBV reference strains that clustered into distinct groups, and corresponded to 77.6 % of the sequences of Mass, 79.3 % of the D274 viruses, 77.0 % of the 793B strain and 76.8 % of the QX strain.

The amino acid alignment of the full-length S1 gene between the IBV/24W, IBV/46W, IBV/62W, H120 and Ma5 vaccine strains is provided in the Supplementary Material (Fig. S1, available with the online Supplementary Material). A comparative analysis of the amino acid sequences deduced from these three IBV isolates with those from Mass and D274 vaccine strains showed that these three Brazilian IBV isolates had two amino acid insertions at positions 120 and 121, and five amino acid insertions at positions 143 to 147 in the S1 protein (Fig. S1).

Based on the full-length S1 gene sequence, IBV/24W showed 98.9 and 97.9 % nucleotide identity with the IBV/46W and IBV/62W isolates, respectively, while IBV/42W shared 98.4 % identity with IBV/62W. The variability among these isolates was mainly characterized by 17 nt (12 aa) and 34 nt (25 aa) substitutions specially located in hypervariable regions (HVR) 1 and 2 of the S1 gene/protein from IBV/46W and IBV/62W isolates, respectively (Table 1). In the pairwise comparison of the S1 gene sequences of IBV24W and IBV46W, pronounced identity was found for the 3′ part of this gene (from 481 nt to 1636 nt), while in the comparison of the isolates IBV46W and IBV62W an identity of 100 % was found for the 5′ part of the S1 gene (first 480 nt).

Homologous and cross virus-neutralization antibody titres

Although some of IBVs in the same genotype exhibited low antigenic relatedness values, the antigenicity of the isolates assessed by viral cross-neutralization assays from the same genotypes in general showed values higher than 146 cross VN end point titres [11, 12].

The results of the virus-neutralization tests showed that the antibody titres measured as geometric mean titres (GMT) between the viruses IBV/24W, IBV/46W, or IBV/62W, and the antibody titres against Mass-vaccine strains (H120 and Ma5) were low, while the GMT ranged from 27 to 49, indicating that all of the isolates possessed a serotype that was distinct from the Mass serotype (Table 2). The neutralization activity of the 24W serum against all three new IBV isolates was also low, and the GMT ranged from 34 to 53. However, this serum showed a high neutralization titre (GMT=368) against the Mass-like serotype. Conversely, the IBV/46W and IBV/62W serum samples showed bidirectional neutralization activity, and the GMT ranged from 203 to 388 against the three Brazilian isolates, and from 293 to 327 against the Mass-like strain. There was a progressive increase in antibody titres against the three IBV isolates, and a maintenance of high neutralization antibody titres against Mass-like strains in the investigated broiler breeders.

Characterization of virulence of IBV isolates by experimental infection of SPF chickens

Mild respiratory clinical signs were observed in all of the experimentally infected SPF chicks from 3 to 6 days post-infection (p.i.), and in the sentinel chickens from 4 to 5 days p.i. in group IBV/62W. The clinical signs included listlessness and ruffled feathers. The birds infected with IBV/24W and IBV/62W showed watery diarrhoea between 8 and 11 days p.i. The clinical signs were more severe in IBV/24W-infected chicks than in those infected with IBV/46W or IBV/62W.

No clinical signs, including macroscopic and microscopic lesions (Fig. 3a, d), were observed in the birds from the
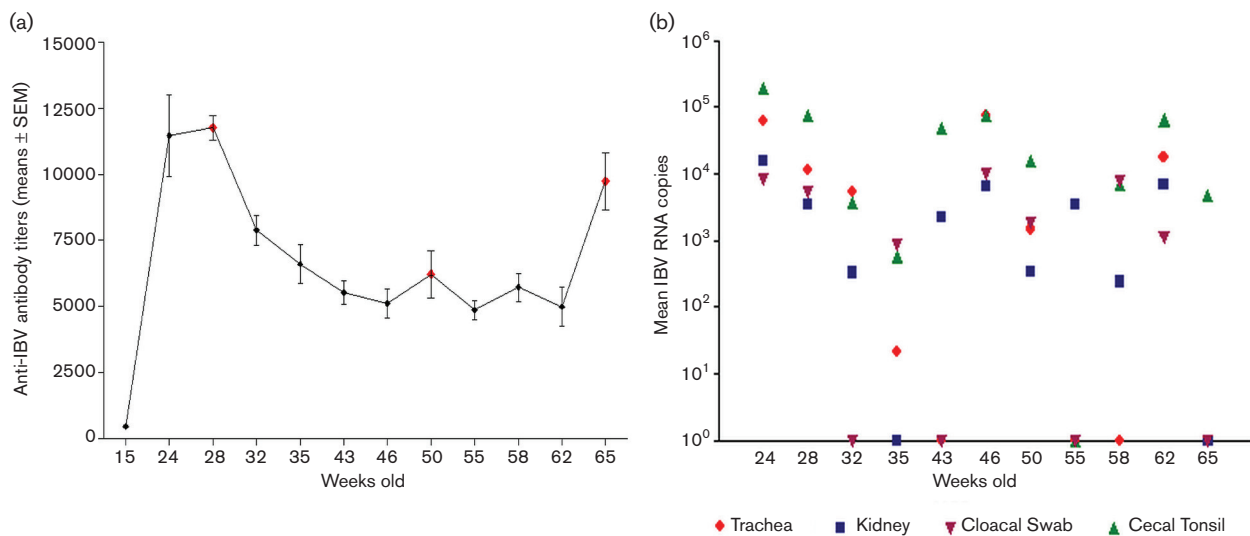

Fig. 1. Assessment of antibody titres and viral load in broiler breeders naturally infected with IBV. (a) Anti-IBV antibody titres detected by ELISA at different ages (weeks) from 15 broiler breeder flocks per interval. Red dots represent post-challenge intervals with clinical manifestations. Error bars indicate the standard error of the mean (means±SEM). (b) Mean viral loads measured by real-time RT-qPCR as the S1 gene copy number present in different tissue samples collected at different time-points from five broiler breeders.

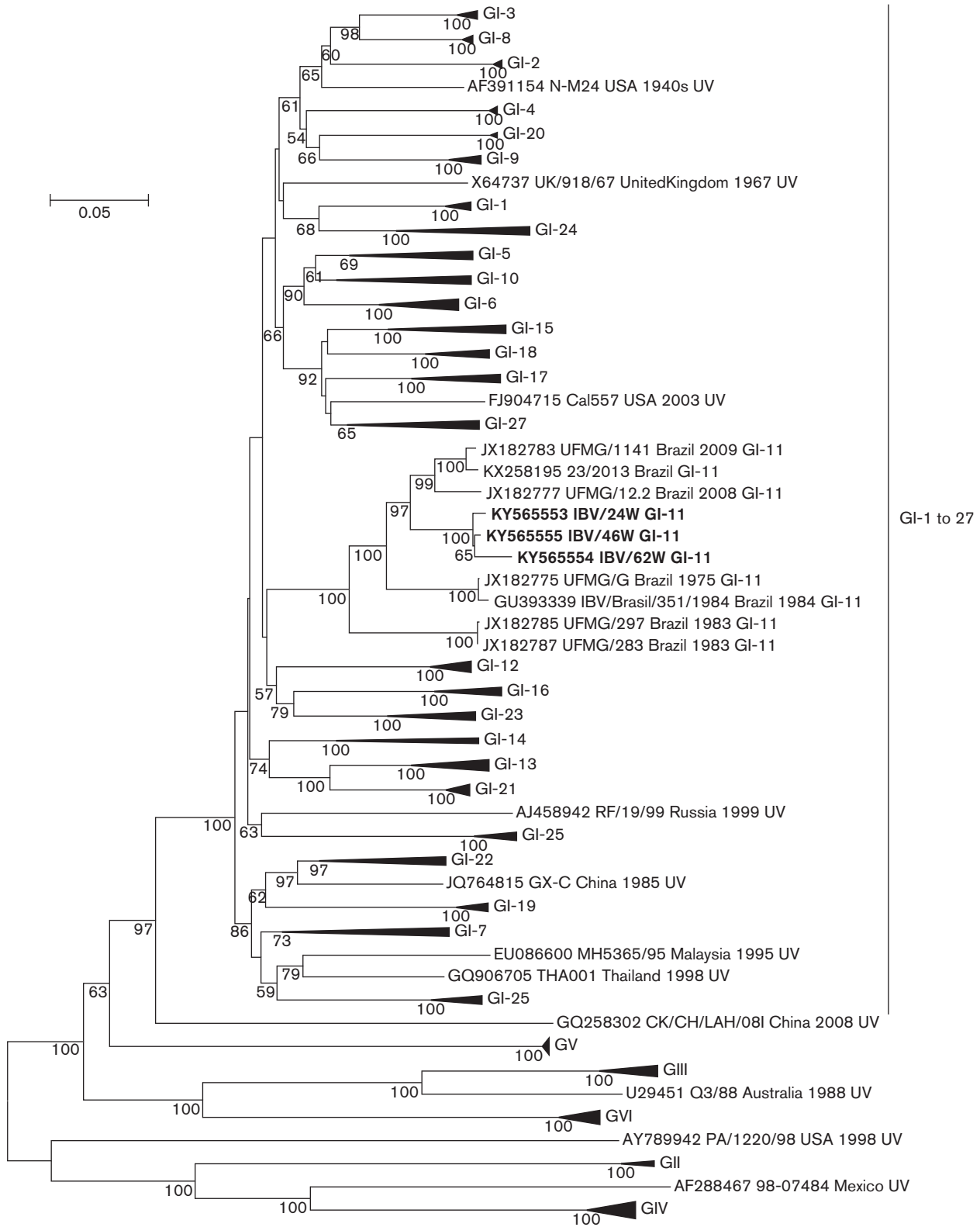**Fig. 2.** Phylogenetic tree of avian infectious bronchitis virus based on the nucleotide sequences of the complete S1 gene constructed using the neighbour-joining method with 1000 bootstrap replicates (bootstrap values are shown on the tree). The IBV isolates sequenced in this study are in bold. The compressed subtrees identify each of the 27 lineages of genotype I, and genotypes II, III, IV, V and VI of IBV. UV, unique variants.

Table 1. Analysis of deduced amino acid sequences of the S1 gene from IBV/24W, IBV/46W and IBV/62W IBV isolates

The sequences were aligned using CLC Sequence Viewer 7.5. The numbers of each alignment show the amino acid positions in the S1 subunit of spike protein for each virus.

Strain	Amino acid position																											
		HVR 1					HVR 2																					
	33	54	57	64	90	102	120	131	158	188	196	203	262	266	270	306	366	367	369	371	385	403	420	475	509	517	519
IBV/24W	A	Q	T	S	T	R	K	N	N	K	R	I	F	R	V	Q	K	Q	V	N	S	G	T	T	G	T	I
IBV/46W	V	H	N	T	M	C	T	D	K	E	R	V	F	R	V	Q	K	Q	V	N	P	G	T	T	G	T	I
IBV/62W	V	H	N	T	M	C	T	D	K	K	H	I	C	G	G	H	R	R	F	Y	P	E	S	M	S	S	L

control and non-infected groups. All three IBV isolates caused mild (Fig. 3b) to moderate (Fig. 3c) microscopic lesions to the trachea of the SPF-infected birds. The major histopathological changes observed in the trachea were characterized by desquamation of ciliated epithelial and mucus-secreting cells, degeneration of epithelial cells and lympho-histiocytic cellular infiltration. In addition, the most prominent tracheal lesions were detected at 4 days p.i. in both groups (Fig. 3c), except for the IBV/24W sentinel birds, which developed the most intense lesions at 7 days p.i. At this post-infection interval, epithelial hyperplasia was observed in the tracheal mucosa, accompanied by slight mononuclear and lympho-histiocytic cellular infiltration.

These IBV isolates also induced interstitial nephritis and tubular epithelial degeneration (Fig. 3e) with moderate intensity at 7 days p.i., especially in the group infected with the IBV/24W isolate and corresponding sentinel chicks. In addition to these lesions, epithelial degeneration and inter-stitial inflammatory infiltration were observed in these sen-tinel birds, especially at 11 days p.i. (Fig. 3f).

Inhibition of tracheal ciliary activity was measured at 4, 7, and 11 days p.i. (Fig. 4). All three IBV isolates caused more intense damage in the trachea at 4 days p.i., and the ciliosta-sis was maintained at high levels at 7 days p.i. The ciliary inhibition returned to minimum scores at 11 days p.i.

The histopathology scores are shown in Fig. 4. Increased tracheal lesion intensity and high viral loads were seen in this organ in experimental infected and sentinel birds with the three IBV isolates at 4 days p.i. However, a higher viral load was detected in kidneys at 7 days p.i., followed by a slight reduction in this parameter at 11 days p.i. As shown in Fig. 4 by RT-qPCR, low to high viral loads were detected at 4, 7 and 11 days p.i. in the trachea and kidney of chickens experimentally infected with isolates IBV/24W, IBV/46W and IBV/62W. Overall, the viral loads and repli-cation efficiency of these viruses in different organs of experimentally infected chickens were very similar. In addi-tion, these isolates showed high affinity to kidney tissues, as reflected by the high number of viral genomic copies detected in this organ.

Regarding the sentinel contact birds, similar scores of histo-pathology and viral load (Fig. 4) were observed in relation to those detected in challenged birds, and these characterize the notable ability of these IBV isolates to spread to suscep-tible contact birds.

DISCUSSION

Understanding the evolution and persistence of IBV in the field of persistently infected broiler breeders is important, especially in the long term, to better elucidate the processes of virus maintenance and circulation in a chicken flock, and also address how this virus is evolving and contributing to the emergence of variant IBV strains that can alter the immune status conferred by regular IBV vaccines. Thus, we evaluated the persistence and biological properties of three

Table 2. Homologous and cross-virus-neutralization titres of avian infectious bronchitis virus isolates and Massachusetts vaccine strain

Viruses	Antisera against IBV			
	24W*	46W*	62W*	Mass[†]
IBV/24W isolate	40[‡]	203	268	49
IBV/46W isolate	34	169	388	38
IBV/62W isolate	53	234	323	27
Mass vaccine strain	368	327	293	266

*Serum samples collected from broiler breeders at different times: 24, 46 and 62 weeks of age.

[†]Anti-serum raised by immunization of SPF chickens with Massachusetts vaccine strains (H120+Ma5).

[‡]GMT of end-point titres for virus-neutralizing anti-IBV antibody.

IBV isolates from different time-points in the productive cycle of a broiler breeder flock. In addition, these viruses were classified in the eleventh lineage of genotype I of IBV, the former BR genotype I, by analysing the full-length sequences of the S1 gene from these isolates and their antigenicity and pathogenicity were characterized.

The current longitudinal analysis showed that the three Brazilian IBV isolates caused relevant pathological alterations in broiler breeders that resulted in increased mortality for these birds in the field, especially during the acute stage of the first episode of infection at 24 weeks, with less severe effects during clinical relapses at 46 and 62 weeks of age. At the beginning of the IBV infection, the affected birds showed low levels of specific neutralizing antibodies, as detected by virus-neutralization (VN) tests against the IBV/24W isolate. In contrast, high VN titres against the Massachusetts vaccine strains were found in these birds at this time-point as a consequence of prior immune stimulation by multi-vaccination with the attenuated and inactivated Massachusetts vaccines used in this flock.

Thus, it can be speculated that the 24-week-old birds that were infected with the variant IBV/24W isolate did not have enough time for seroconversion against the BR genotype I IBV after infection with a viral strain of this genotype. The main reason for this is the reduced identity between the amino acid sequence of the S1 protein from the IBV/24W isolate and that of the Massachusetts vaccine strain, while most of the mutations affected amino acid residues belonging to the structure of the VN epitopes of this protein, resulting in lower cross-neutralization between these viruses, as has been reported to occur for other IBV strains or isolates [26, 27].

Indeed, the S1 glycoprotein carries the major neutralizing epitopes of IBV, and the corresponding S1 gene is highly variable, which may lead to the emergence of new genotypes or serotypes [11, 12]. In spite of this, the 3-aa difference in the HVR1 region, and another 3-aa difference in the HVR2 region between the isolate IBV/24W and the isolates IBV/46W and IBV/62W did not cause any relevant reduction in

the cross-neutralization activity against the IBV/24W isolate from the antisera collected at 46 and 62 weeks of age from broiler breeders in the field. Furthermore, previous studies have shown that in some circumstances mutations in any of the three HVRs of the S1 gene from different IBV strains cannot affect the antigenicity of these viruses [12]. The cross-neutralization ability of 24-week anti-serum samples against the three IBV isolates was lower than that of the 46-week and 62-week anti-serum samples. Thus, there is low protection against IBV infection and disease for breeders at 24 weeks of age, as expressed by the high viral loads and histological lesions found in these birds. However, the Massachusetts vaccination elicited high antibody titres, which were detected in the commercial ELISA test and the VN test against this IBV strain.

In fact, the IBV/24W isolate that caused the first episode of IBV infection in the broiler breeder flock investigated in this study showed 77.6 and 79.3 % amino acid identity compared with the S1 gene of vaccine strains Ma5 and D274 IBV, respectively. It is well known that cross-protection is poor between different serotypes and genotypes of IBV strains that have less than 85 % identity in the amino acid sequences of their S1 protein [8, 9, 28], suggesting that the IBV Massachusetts vaccines used in this flock may not confer full cross-protection against these variant IBV isolates.

We can assume that following infection by the IBV/24W isolate, the virus has established itself among the broiler breeders of this flock and reached a persistent status, despite the presence of high neutralizing antibody titres against these Brazilian variant viruses in the 46- and 62-week-old birds. Thus, these anti-BR-IBV antibodies that are present in the blood serum seem to exert a partial protective role for the broiler breeders against the most severe clinical and pathological manifestations caused by the IBV/46W and IBV/62W isolates. However, these systemic anti-IBV antibodies were not as effective as other putative local immune responses in impairing IBV infection by these isolates in persistently infected organs of the breeder chickens, especially in view of the role of mucosal antibodies of the IgA and IgG isotypes, and the cytotoxic responses mediated by the TCD8+ cells. Moreover, it is noteworthy that these local immune responses, if activated, may exert relevant roles against IBV during a persistent infection [29, 30], but if they are not activated, they may favour viral escape and persistence [31]. Nevertheless, we did not evaluate the local immune responses in the target organs of IBV replication during its persistence, such as cecal tonsils, intestine and kidney, and it is important to investigate these parameters further in breeder chickens.

The IBV genome was detected in chickens experimentally infected with IBV/24W, IBV/46W and IBV/62W isolates at high viral loads in cecal, tonsil and cloacal swab samples throughout all of the productive cycles of broiler breeders. It is important to consider that during an active IBV infection, in the absence of clinical disease, and during the course of
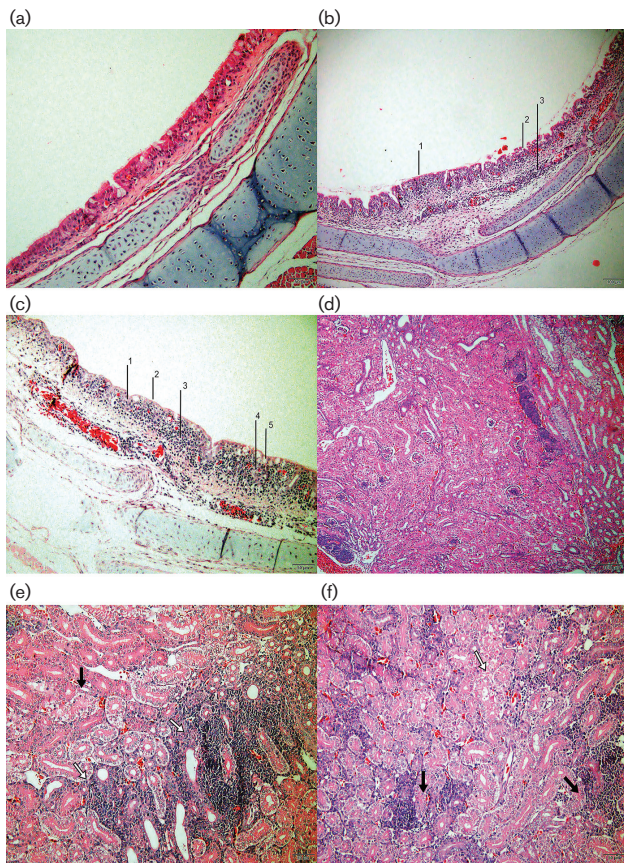
Fig. 3. Hematoxylin and eosin stain (H and E) of tissues from chickens experimentally infected with IBV isolates (IBV/24W, IBV/46W and IBV/62W). Photomicrographs (a) and (d) correspond to non-infected control tissues. (b) Trachea with deciliation in the epithelium [1], degeneration and necrosis of some epithelial cells [2], infiltration of lymphocytes and heterophils in the epithelium and lamina propria [3] in the chicken challenged with IBV/46W. (c) Trachea from chicken challenged with the IBV/62W showing moderate diffuse lymphocytic infiltration and hyperemia of lamina propria [3, 4] at 4 days p.i., with the presence of epithelial cells, necrosis and loss of glandular structure [5], mild hyperplasia [1] and desquamation of ciliated epithelial cells [2]. (e) Tubulo-interstitial lymphocytic infiltrates (white arrow) and mild degeneration of tubular cells (black arrow) from infected chicken with the IBV/24W isolate at 7 days p.i. (f) Multifocal lymphocytic infiltration (black arrow) with the presence of degenerated cells and heterophils in the lumen and with mild degeneration of tubular cells (white arrow) from sentinel chicken challenged with IBV/24W at 11 days p.i.

chronic and persistent infections, faeces and the gastro-intestinal tract contain a significant amount of virus, and persistently infected tissues are a likely source of reinfection in layers/breeders, which can occur at regular intervals, as reported previously [32–36].

The tissue damage caused by the experimental infection with the three IBV isolates was evident in the trachea and kidney, and coincidently, the viral loads were higher in these two organs. The viral RNA levels in the tracheas of challenged and sentinel birds with IBV isolates declined

after peaking at 4 days p.i., while in the kidney, they peaked at 7 to 11 days p.i., or soon after the contact birds were placed in the challenged group. Similarly to the results of this study, some IBV strains have been documented to reach peak IBV loads in the trachea within 3 to 5 days p.i., and to remain at these levels for 7 days [22, 29, 37, 38].

In recent years, the pathotypes and protectotypes of Brazilian variant IBV strains have been investigated by the experimental infection of SPF chickens [4, 39]. Some of these strains were characterized as nephropathogenic and less pathogenic for tracheal tissue, and they were only partially cross-protected by the Massachusetts vaccines [4, 39]. Indeed, the nephropathogenic IBV strains of different serotypes have been one of the main health problems for poultry worldwide [27, 28, 40], and the nephropathogenic strains, along with other IBV strains, are continuously evolving and producing new IBV variants for which the majority of reference vaccine strains do not offer cross-immunity [41].

Despite the occurrence of 12 amino acid substitutions between IBV/24W and IBV/46W isolates, there were no drastic alterations in the tissue tropism or pathogenicity of these viruses when they were tested through experimental infection in SPF birds. This indicates that these amino acid changes did not affect the receptor-binding domain of the S1 protein of these IBV isolates, in particular for interaction with $\alpha-2,3$-sialic acid or other putative IBV receptors on the surface of the cells of the respiratory and urinary tracts of the chicken host [42, 43]. Thus, the S1 protein of these IBV isolates does not seem to be the only factor responsible for the expression of viral pathogenicity.

Although RNA viruses, such as the avian infectious bronchitis and influenza viruses, are present in biological samples as a group of closely related variants (quasispecies), the nucleotide sequences of the more variable genes of these viruses, as determined by the Sanger method, more frequently identify the predominant genetic sequence among the group of variants present in one isolate [21, 44, 45]. Thus, despite the fact that we did not determine the frequencies of each variant subpopulation of the IBV isolates in this study, we assumed that these three isolates might constitute distinct variant subpopulations. In addition, these three variants may be the most prevalent among the group of variants generated by the viral replication process and shaped by immune selection pressures in persistently infected hosts, as demonstrated by the nucleotide sequences of the S1 gene of the IBV isolates obtained at 24, 46 and 62 weeks of age from broiler breeders affected by these viruses, which were determined using the Sanger method. This hypothesis is supported by the findings of Toro *et al.* [46], which show that the Ark-type IBV virulent strain is subjected to immune selection by the infected chicken host, as well being affected by the selection process acting on Ark-type vaccine strains [23, 47].
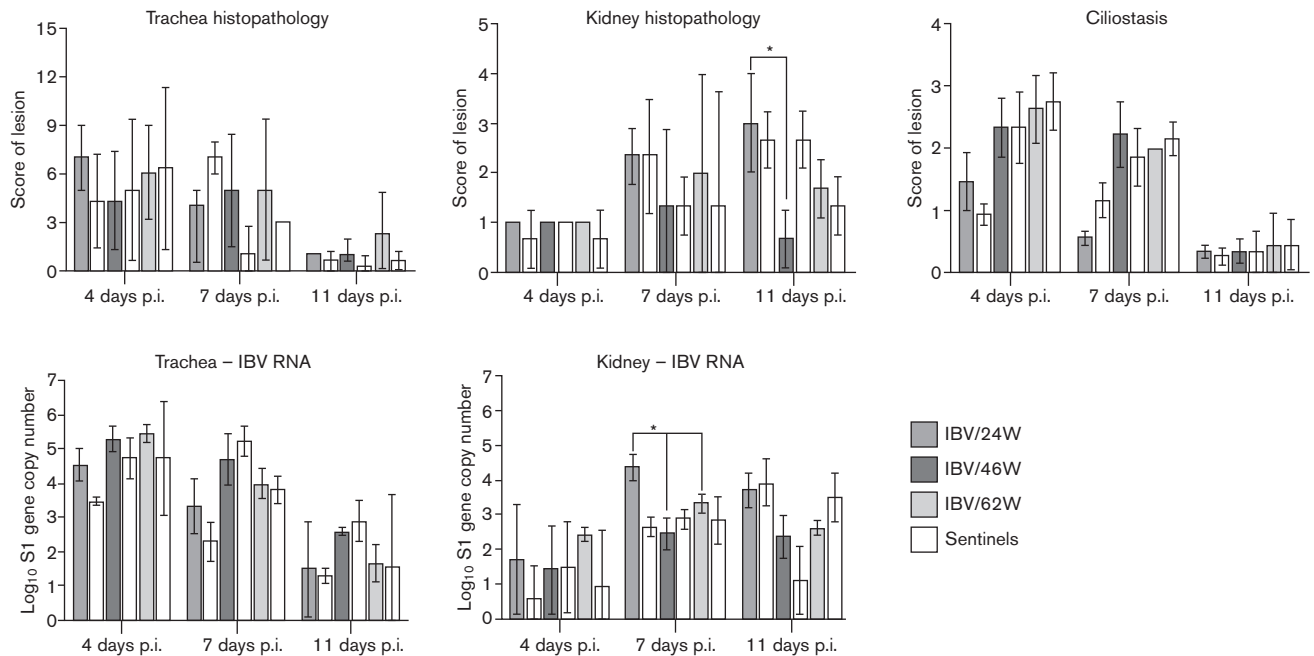
Fig. 4. Tracheal and kidney pathological alterations, measured as tracheal ciliostasis scores, histopathology scores and viral loads induced by experimental infection of 14-day-old SPF chickens with IBV isolates (IBV/24W, IBV/46W and IBV/62W). Intervals of 4, 7, 11 days p.i. were analysed. Quantification of IBV is expressed as the $\log_{10}$ of the number of copies of the S1 gene in trachea and kidney that were measured by real-time reverse transcription-PCR with SYBR Green I. (*$P<0.05$.)

As three outbreaks of infectious bronchitis occurred in broiler breeders that had been multi-vaccinated with Massachusetts strains at 24, 46 and 62 weeks of age, despite the presence of high VN antibody titres against this vaccine IBV strain at the same time-points, we concluded that the vaccination programme adopted for these birds did not prevent the persistence of IBV infection or the development of the disease in these birds.

By contrast, field variant IBV isolates were still able to cause acute disease episodes followed by persistent infection in broiler breeders, in which these viruses seemed to undergo continuous genetic evolution, resulting in the generation of progenies of IBV variants, and complicating the effective control of this infection. The genetic alterations found in the S1 gene and the corresponding protein detected in these three IBV isolates obtained from each outbreak involving broiler breeders are not able to change the tissue tropism and pathogenicity of these viruses in the experimental infection of SPF birds, but seem to negatively influence the effectiveness of the host immune-protection responses against these viruses and favour viral persistence, despite the presence of high levels of systemic VN antibodies against these IBV isolates from 46 weeks of age onwards in these broiler breeders.

Finally, all of the information gathered by this study will contribute to our further understanding of the current situation in Brazil, with regard to new and evolving variant IBV strains appearing in commercial poultry with long production cycles, such as broiler breeders.

METHODS

History and sample collection

For 42 weeks, three flocks of broiler breeders housed in poultry farms located in southern and southeast Brazil were evaluated after showing clinical signs strongly indicative of IBV infection. All three flocks were vaccinated at 1 (ocular route), 4 and 8 weeks of age (through drinking water) with a live attenuated vaccine formulated with the Massachusetts (Mass) strain, followed by vaccination at 12 weeks of age with an inactivated vaccine (intramuscularly route) containing Mass and D274 IBV strains. Both flocks were vaccinated periodically with attenuated Mass vaccine by drinking water at 8-week intervals from 16 weeks of age onwards (16, 24, 32, 40, 48 and 56 weeks of age).

These flocks developed characteristic clinical signs of IBV infection between 23 and 24 weeks of age, when the chickens were entering the beginning of production and soon after their transfer from the rearing to the production premises. Five birds identified with suspected IBV infection were euthanized for the collection of kidney, trachea, cecal tonsil and cloacal swab samples. These samples were submitted to the extraction of RNA for analysis by RT-qPCR and virus isolation. In addition, serum samples were collected from 15 birds for ELISA testing. After the diagnosis of Brazilian IBV

genotype in these breeder flocks, samples were taken at intervals that ranged from 3 to 8 weeks, up to the end of their productive cycle at 65 weeks of age.

IBV diagnosis

The diagnosis was performed for viral detection, after clinical suspicion in the field, by a duplex-RT-qPCR for the detection of the IBV universal sequence of the 3′-UTR gene and the S1 gene sequence of the Massachusetts genotype [48]. The samples that only showed amplification of the 3′-UTR gene were selected as non-Massachusetts genotypes and subjected to virus isolation in embryonated eggs. The trachea and kidney samples from which these IBV strains were obtained were also processed for histopathological examination.

Virus isolation and titration

Tissues and swabs samples were prepared at 20 % (w/v) in PBS, 0.01 M, pH 7.2, clarified by centrifugation at 12 000 r.p.m. for 20 min at 4 °C, added to 100× concentrated antibiotics (Sigma-Aldrich, St Louis, MO, USA), filtered through 0.22 µm membranes and inoculated as described previously [49]. Both the IBV vaccine strains and the isolates were titrated in embryonated chicken eggs, and the 50 % embryo-infective doses (EID_{50}) were determined by the Reed and Muench method [50].

RNA extraction and absolute quantification of viral load

The RNA was extracted from tissue homogenates using an RNeasy mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Absolute quantification of viral load was performed using oligonucleotides for the S1 gene of IBV [51], and the Ct results were used to calculate the $\log_{10}$ of the number of moles using the linear equation from the standard curve, as optimized previously [29], with the exception that the open reading frame of the S1 gene of the Massachusetts-41 strain sequence (GenBank accession number AY561711.1) was cloned into the pIDTSmart vector (Integrated DNA Technologies, USA). One-Step-RT-qPCR analyses were run on a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA).

The reaction was performed with the QuantiFast SYBR Green RT-PCR kit, plus RNase inhibitor (Qiagen, Hilden, Germany) for a final volume of 20 µl/reaction, in accordance with the manufacturer's instructions.

Reverse-transcription PCR and DNA sequencing of the complete S1 gene of IBV

Reverse transcription-PCR (RT-PCR) was performed on the RNA extracted from AF fluid infected with IBV isolates using the OneStep RT-PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A set of the primers S1/S2 and S5/S4 was used to amplify the complete S1 gene sequence, and the annealing temperatures were used according to the recommendations previously described [20].

The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and nucleotide sequencing from both strands of amplified DNA was performed with the BigDye Terminator version 3.1 cycle sequencing kit, while products were analysed on the ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis and estimation of evolutionary distances

The alignment, consensus sequence and comparison of the nucleotide and amino acid sequences between the isolates and selected strains representing established IBV genotypes were performed using the Clustal method with the BioEdit sequence alignment editor [52].

Phylogenetic analysis was performed using MEGA version 6.0 [53]. The S1 gene sequences of the IBV isolates were analysed using 121 sequences of the full S1 gene from genotypes I (lineage 1–27), II, III and IV, as published in GenBank, and classified based on the method proposed [54]. Information on the selected sequences is provided as Supplementary Material (Table S1). The evolutionary history was inferred using the neighbour-joining method, and the evolutionary distances were computed using the maximum composite likelihood method, with standard errors being calculated based on 1000 bootstrap replicates and presented as the number of base substitutions per site. The names and numbers of the S1 gene sequences of the IBV strains used in the phylogenetic tree represent the names and accession numbers in GenBank.

Serological monitoring of anti-IBV antibodies

Serum samples collected from 15 birds that showed clinical–pathological characteristics of IBV infection in the field were tested in duplicate for the presence of anti-IBV antibodies by a commercial ELISA kit (IDEXX Laboratories, Westbrook, ME, USA). The endpoint anti-IBV antibody titres were calculated, and titres>396 were considered to be positive for IBV antibody, according to the manufacturer's instructions.

Virus neutralization

Chicken kidney primary cell cultures (CKCs) obtained from day-old SPF were used for virus propagation and virus neutralization testing. The CKC-adapted IBV was obtained from AF containing the IBV/24W, IBV/46W and IBV/62W isolates, and grown in CKC monolayer cultured in HAM-F10 (Sigma-Aldrich, Saint Louis, MO, USA) and 199 modified medium (Nutricell, Campinas, Brazil) supplemented with 4 % foetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) using 60 mm ×15 mm tissue culture plates (Corning, USA) and incubated at 38 °C and 5 % CO_2. Virus titre was determined by microtitration each time that the passages were performed, and the results were expressed as the $TCID_{50}$ ml^{-1}.

The constant-virus diluting-serum microneutralization test was performed in 96-well microplates (Corning, USA), as

described by Blore and Skeeles [55]. Briefly, for the VN test, equal volumes of 10^2 $TCID_{50}$ of the CEK-adapted IBVs and serial twofold dilutions of 15 antisera (2 wells for each dilution) collected from naturally infected chickens were mixed. Then, $100\,\mu l$ of the virus–antisera mixture was transferred to CKC cell cultures. The plates were incubated for 96 h, and the 50 % end-point neutralizing titres were calculated using Reed and Muench's method [49]. The geometric mean titre (GMT) was determined by the 'ready reckoner' method [56].

At 8 weeks of age, the SPF chickens maintained in positive-pressure isolators were inoculated via the ocular-nasal route with $10^{5.0}$ EID50%/100 ul of both the H120 (Merial, Brazil) and Ma5 (Intervet, Netherlands) strains to produce a mono-specific antiserum against the Massachusetts strains. Three weeks later, the chicken were inoculated with $10^{4.0}$ EID_{50} by the intratracheal and intravenous route. After 3 weeks, the chicken were inoculated via the subcutaneous route with the same strains emulsified with Montanide ISA 71 VG (SEPPIC, Puteaux, France) according to the manufacturer's instructions. Blood samples were obtained 3 weeks after the last inoculation, and serum was harvested and inactivated at $56\,°C$ for 30 min and stored at $-20\,°C$.

Virus challenge and assessment of pathogenicity of IBV isolates

Four groups of 12 SPF chicks (White Leghorn lineage) were used. The birds were housed in positive-pressure isolators. At 14 days of age, groups IBV/24, IBV/46 and IBV/62 were experimentally infected via the intra-ocular and intranasal routes with isolates of IBV ($10^{4.0}$ EID_{50}/bird). A negative control group was mock-infected with SPF AF and maintained under the same conditions. Three chickens from each group were sacrificed at 4, 7 and 11 days p.i., and trachea, kidney, cecal, tonsil and cloacal swabs were collected. Tracheal and kidney samples were collected from each group; a portion was immediately frozen and kept at $-70\,°C$ until processing, and the remaining portions were subjected to histopathological processing. Additionally, a portion of trachea was submitted for ciliostasis analysis.

To assess the viral spread of the IBV isolates, 14-day-old sentinel SPF chicks were inserted into all challenged groups in order to expose the birds to the challenged ones. Sentinel chicks were inserted 4 days after challenge and were sacrificed at 4, 7 and 11 days post-contact before being submitted to the same sampling procedures as the challenged groups. All of the animals used in this study were cared for in accordance with established guidelines, and the experimental protocols were performed with the approval of the Institutional Ethics and Animal Welfare Committee (CEUA, Unesp; protocol number 4021/15).

Histopathology

Samples of kidney and 0.5 cm of the medial part of each tracheal third were placed in 10 % (v/v) buffered formalin (pH 7.2). Next, the fixed fragments were dehydrated, diaphanized, embedded in paraffin, sectioned at $5\,\mu m$ and stained with hematoxylin and eosin.

The slides were examined by light microscopy, and the lesion scores ranged from 0 to 3. Absence of injury was classified as 0, while mild, moderate and severe injuries were classified as 1, 2 and 3, respectively [57, 58]. Loss of cilia and degeneration of epithelial cells, glandular degeneration of the epithelium, inflammatory infiltration, epithelial hyperplasia and inflammatory cell infiltration in the tunica adventitia were evaluated. The maximum score for tracheal lesions was 18. Histopathological analysis of the kidneys was performed according to the parameters described by Chen *et al.* [58]. In summary, changes were observed as epithelial degeneration, interstitial inflammatory and inflammatory infiltrate in the ureter. The maximum score value was 9.

Ciliary activity inhibition

The whole trachea was removed with sterile forceps and scissors immediately after the chickens were sacrificed. Three rings from the upper, middle and lower part of the trachea were cut and placed into minimal essential medium (MEM; Sigma-Aldrich, Saint Louis, MO, USA) plus 5 % FBS (Gibco, Grand Island, NY, USA) in individual plates. The rings were then examined under low-power magnification and scored [59].

Statistical analysis

The comparisons of viral load and microscopic lesions between the experimental groups were performed using the Kruskal–Wallis test. All analyses were conducted using the GraphPad software (version 4.0), and the probability level for significance was set at $P \leq 0.05$.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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