

## Article

# Molecular biology and pathological process of an infectious bronchitis virus with enteric tropism in commercial broilers

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**Abstract:** Infectious bronchitis virus (IBV) induces respiratory and urogenital disease in chickens. Although IBV replicates in the gastrointestinal tract, enteric lesions are uncommon. We have reported a case of runting-stunting syndrome in commercial broilers from which an IBV variant was isolated from the intestines. The isolate, CalEnt, demonstrated an enteric tissue tropism in chicken embryos and SPF chickens experimentally. Here, we determined the full genome of CalEnt and compared it to other IBV strains, in addition to comparing the pathobiology of CalEnt and M41 in commercial broilers. Despite the high whole-genome identity to other IBV strains, CalEnt is rather unique in nucleotide composition. The S gene phylogenetic analyses showed great similarity between CalEnt and Cal 99. Clinically, vent staining was slightly more frequent in CalEnt-infected birds than those challenged with M41. Furthermore, IBV IHC detection was more evident and the viral shedding in feces was overall higher with the CalEnt challenge compared with M41. Despite underlying intestinal lesions caused by coccidiosis and salmonellosis vaccination, microscopic lesions in CalEnt-infected chickens were more severe than in M41-infected chickens or controls, supporting the enteric tropism of CalEnt. Further studies in SPF chickens are needed to determine the pathogenesis of the virus, its molecular mechanisms for the enteric tropism, and its influence in intestinal health.

**Keywords:** IBV, infectious bronchitis, variants, whole-genome sequencing, enteric tropism, runting-stunting syndrome

## 1. Introduction

Infectious bronchitis virus (IBV) belongs to the Gammacoronavirus genus and mainly causes respiratory and urogenital disease in chickens. Although IBV replicates and persists in the cecal tonsils and has been isolated from duodenum and jejunum, intestinal lesions are rare [1-3]. Genetic variation of the viral spike (S) gene may lead to an increased susceptibility to proteolytic activation and to an improved ability to bind cell receptors, enhancing its infectivity. In addition, genomic changes modulate the virus entry and influence tissue tropism, persistence, virulence and host range [4]. This variability explains why coronaviruses genetically and antigenically similar to IBV can cause different clinical outcomes. For example, the turkey coronavirus (TCoV) induces severe enteric disease by the viral replication in enterocytes of the jejunum and ileum [5-7]. Molecular differences between TCoV and IBV are mainly found in the S gene, in which

homologies are as low as 33%, validating the divergent tissue tropism of these closely related viruses [8].

An enterotropic IBV strain has been described for causing intestinal lesions, mainly in the rectum, consisting of desquamation of epithelial cells on the villi tips and congestion of the intestinal submucosa [9,10]. This virus was originally isolated from the intestines of chickens presenting with respiratory signs [11]. In another study, an IBV strain isolated from broilers with enteric and respiratory diseases was compared to an isolate from broilers that prompted only respiratory disease. Both isolates induced similar pathology after experimental infection; both viruses were detected in the intestines but their infection did not cause enteric lesions [12]. IBV was also one of several pathogens isolated from the intestines of commercial broilers presenting with runting-stunting syndrome. Infection with these IBV isolates alone or combined with other microorganisms induced a reduced body weight in experimentally infected chickens [13].

Previously, we have described the detection and isolation of an IBV variant from the intestines of brown broiler chickens showing runting-stunting signs. Interestingly, the virus was not retrieved from kidneys or respiratory tract. The enteric isolate was named CalEnt and was isolated from intestines of chicken embryos, but not from the chorioallantoic membrane. Additionally, CalEnt induced runting-stunting syndrome-like lesions after experimental oculonasal infection of specific-pathogen free (SPF) chickens. The S1 gene sequence of CalEnt showed nucleic acid sequence identities of 93.8% to IBV California 99 (Cal 99) and of 85.7% to IBV Arkansas DPI (Ark DPI) [14]. The aim of the present investigation was to determine the full genome of CalEnt and compare its genomic identity to other IBV strains, as well as to compare the pathobiology of CalEnt to that of the respiratory strain M41 after a controlled infection of commercial broilers.

## 2. Materials and Methods

### 3.1. Viruses

IBV CalEnt and M41 strains were used in experimental challenges. Both IBV strains were grown and titrated in embryonated chicken SPF eggs (Charles River, CT, USA) using standard procedures [15,16]. The viral dose of IBV CalEnt was  $2 \times 10^4$  EID<sub>50</sub> and the dose of IBV M41 was  $2 \times 10^5$  EID<sub>50</sub> in a 200- $\mu$ L inoculum. The challenge was performed either oculonasally or via crop gavage.

### 3.2. Whole genome sequencing

Intestines from embryos infected with CalEnt were homogenized with PBS and RNA was extracted from a volume of 100  $\mu$ L using TRIzol (ThermoFisher, Waltham, MA, USA). DNA depletion was performed using the Turbo DNA-Free Kit (ThermoFisher, Waltham, MA, USA) followed by rRNA depletion using the Terminator 5'-Phosphate-Dependent Exonuclease (Epicentre Biotechnologies, Madison, WI, USA) as per the manufacturer's instructions. The DNase reaction was ceased by adding EDTA at a concentration of 5 mmol/L. The RNA was purified using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) without the addition of the carrier RNA, and eluted to a final volume of 30  $\mu$ L. The RNA quality was evaluated using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). cDNA libraries were prepared using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). Libraries were sequenced using the Illumina at 100-bp paired end. The sequences were assembled using IBV Cal99 as a reference and the consensus was uploaded to GenBank (accession number MW556742).

The obtained consensus sequence was aligned with several IBV variants and TCoV using the MAFFT plugin [17] in Geneious Prime 2020.1.1. Nucleotide sequences were used to calculate homologies and for phylogenetic analyses using the maximum-likelihood method based on the GTRGAMMAI model with 1,000 bootstraps in Geneious Prime with the RaxML plugin [18]. Homology matrices were calculated, and phylogeny was performed in whole genomes, full S gene sequences and 751-bp fragments of the S gene

bearing the S1 hypervariable region (nucleotide positions 20,368 to 21,119 of GenBank accession number MW556742).

3.3. Experimental design

Two hundred one-day-old commercial broilers were obtained from a commercial hatchery. The birds were vaccinated at the hatchery against coccidia and *Salmonella*, but not for IBV. The chickens were divided into 5 groups of 40 birds each (Table 1) and housed in BSL2 rooms at the Teaching and Research Animal Care Services at the University of California, Davis. Feed and water were provided *ad libitum*. All animal experimental procedures were approved by the University of California, Davis Institutional Animal Care and Use Committee (Approval No. 19092). In the first week of life, 2 birds from group 1, one bird from group 2 and one bird from group 3 deceased from unspecific reasons unrelated to IBV (Table 1).

**Table 1.** Experimental groups composed of commercial broilers unchallenged or challenged with infectious bronchitis virus strains with enteric (CalEnt) and respiratory (M41) tropisms.

Group	Virus strain	Infectious route	No. of birds at challenge <sup>1</sup>
1	CalEnt	Crop gavage	38
2	CalEnt	Oculonasal	39
3	M41	Crop gavage	39
4	M41	Oculonasal	40
5	Uninfected	---	40

<sup>1</sup> Four birds deceased prior to the virus challenge

At 10 days of age, blood was collected from 10 birds of each group via wing vein puncture and tested for maternal antibodies. When 14 days old, four groups were inoculated with 200 µL of either one of the viruses directly into crop using a buttoned cannula or via oculonasal route. The fifth group was left unchallenged as a negative control (Table 1).

Respiratory signs were evaluated at 4, 6, 10 and 14 dpi. Individual respiratory sign scores were recorded as 0 (no signs), 1 (mild nasal rales or upper respiratory tract sounds), 2 (moderate tracheal rales) or 3 (severe respiratory sounds audible from a 20-cm distance) by a blinded investigator [19]. On the same days, the presence or absence of vent stains due to diarrhea was recorded.

At one, 2, 4, 6, 10 and 14 dpi, tears and cloacal swabs were collected from five chickens per group for detection of IBV by RT-qPCR. Tears were collected using sodium chloride to stimulate lachrymation. Tracheas and small intestine samples collected during necropsies at 4 and 14 dpi were submitted for histopathology and immunohistochemistry (IHC).

3.4. Serology

Sera collected at 10 days of age were tested for antibodies against IBV by ELISA (IDEXX Laboratories, Westbrook, ME, USA). S/P ratios were transformed into titers as per the manufacturer’s recommendations.

3.5. RT-qPCR

RNA was extracted from tears and cloacal swabs in PBS using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) as described by the manufacturer. A probe-based RT-qPCR using primers qIBf (ATA CTC CTA ACT AAT GGT CAA CAA TG) and qIBr (GGC AAG TGG TCT GGT TCA C) and probe qIBs (CAA GCC ACT GAC CCT CAC AAT AA) was performed, targeting a 134-bp fragment of the IBV M gene in 44 cycles of denaturation and combined annealing/extension steps [20,21]. Viral load was expressed

as the total number of total cycles (44) – Cq. The relative difference between viral load in tears and cloacal swabs was calculated as the difference of the mean viral loads.

### 3.6. Histopathology and immunohistochemistry

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm, mounted on glass slides and stained with hematoxylin and eosin (H&E) stains. IBV *in situ* antigen detection was performed using IHC staining as previously described [22]. A combination of two monoclonal antibodies targeting the IBV matrix and spike proteins were used [23,24].

### 3.7. Statistical analyses

Viral loads (represented as 44-Cq) were compared using ANOVA followed by Tukey's multiple comparison tests. Respiratory sign scores, vent staining and uniformities were compared using Kruskal-Wallis (for non-parametric data) followed by Dunn's multiple comparison tests. Data was analyzed using Prism 9 (GraphPad, La Jolla, CA, USA).

## 3. Results

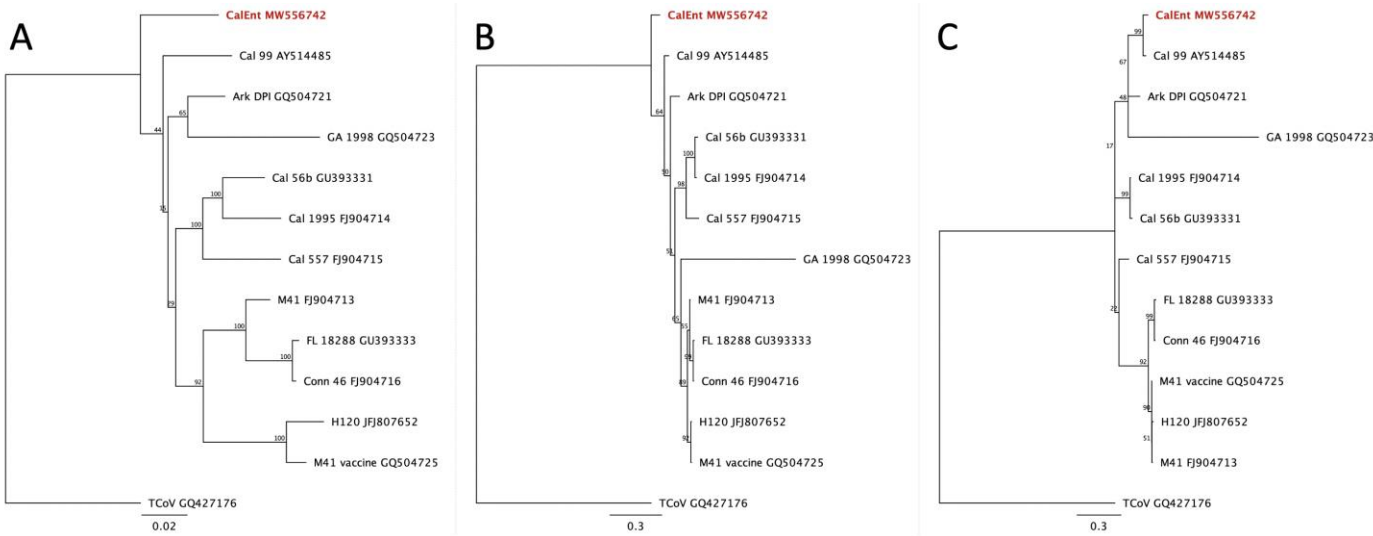
### 3.1. Whole genome analyses

The complete genome homologies revealed that the IBV strains most closely related to CalEnt were Cal99 (94.3%), Cal 56b (94.2%), M41 (94.1%) and ArkDPI (94.1%) (Table S1). The whole genome comparisons demonstrate that the highest genetic variability is in the S gene, where the nucleotide homologies to CalEnt was the lowest and averaged 81.1% (Table 2). Considering the full S gene, CalEnt was most similar to Cal 99 (88.4%), followed by Ark DPI (84.6%), Cal 1995 (83.4%) and Cal 56b (83.2%) (Table S2). When focusing on the hypervariable region of the S1 gene, CalEnt shows a strikingly high homology to Cal 99 (95.5%) (Table S3).

**Table 2.** Percent nucleotide identity between infectious bronchitis virus CalEnt strain and other relevant IBV strains. The highest spike (S) gene homologies are bolded and colored. The genes are sorted from lowest to highest average percent identity.

Gene	Ark DPI	Cal99	Cal 557	Cal 56b	Cal95	Conn46	FL18288	M41	H120	M41vac	GA98	TCoV
<b>Spike</b>	<b>84.6</b>	<b>88.4</b>	83.6	83.2	83.4	80.47	80.2	80.8	80.8	80.8	67.2	47.5
<b>Envelope</b>	91.6	90.4	80.2	77.2	79.0	91.89	90.1	91.0	86.5	86.5	88.6	88.0
<b>3a</b>	97.1	90.2	93.1	89.7	90.2	90.23	89.7	90.2	85.1	85.1	94.8	89.7
<b>Matrix</b>	95.2	95.3	91.6	89.1	89.3	95.15	95.0	95.2	90.5	90.3	89.7	94.3
<b>3b</b>	96.9	96.4	91.3	93.3	91.3	97.44	96.4	97.4	83.1	82.8	94.4	95.9
<b>1a</b>	94.3	93.8	94.2	95.6	94.7	93.77	93.7	95.6	93.8	93.5	95.6	94.1
<b>1ab</b>	95.1	95.2	95.1	96.3	95.3	95.34	95.3	95.8	93.8	93.9	96.0	95.0
<b>5a</b>	98.0	98.0	96.7	91.4	89.9	97.98	97.5	98.0	92.4	93.7	97.5	95.0
<b>Nucleocapsid</b>	98.7	95.1	94.5	98.7	94.1	99.07	98.7	99.1	93.4	95.2	99.0	95.3
<b>5b</b>	98.4	98.0	97.6	98.8	98.8	99.20	99.2	98.4	97.2	97.8	99.2	96.4

Despite the similarities to Cal 99, Cal 56b, M41 and Ark DPI, CalEnt did not cluster with other IBV strains in the whole genome phylogenetic analysis (Figure 1A). Similarly, the full S gene phylogenetic tree shows CalEnt relatively distant from other IBV strains (Figure 1B), indicating its unique nucleotide composition. The S1 gene hypervariable region phylogeny corroborates the high homology between CalEnt and Cal 99, demonstrating that these two strains cluster together (Figure 1C).



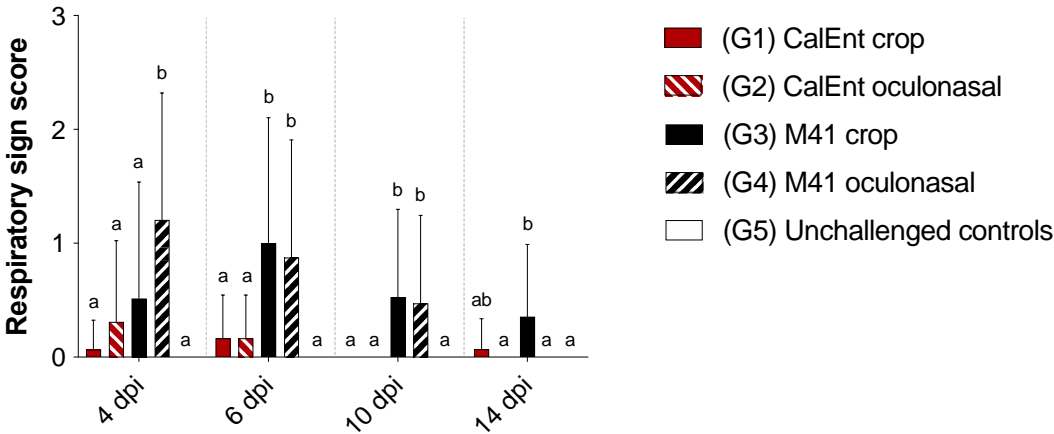
**Figure 1.** Phylogenetic analyses of the whole genome (A), the full S gene (B) and the hypervariable region of the S gene (C) of infectious bronchitis virus isolates. The maximum likelihood method with 1,000 bootstrap replicates was used.

3.2. Maternal antibodies

IBV maternal antibodies were detected in 10% of the collected serum samples (n=50) at 10 days of age. The geometric mean titer and standard deviation was 172±166.

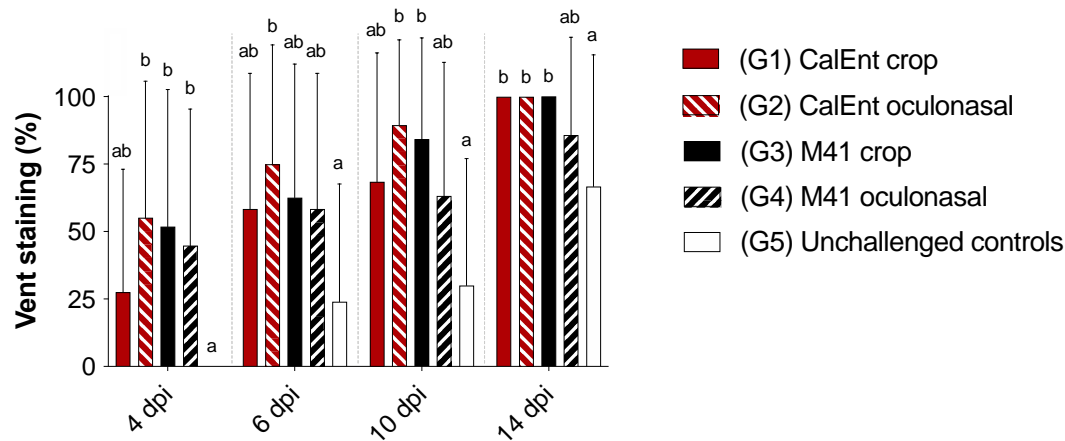
3.3. Clinical signs

Overall, M41-infected birds presented with more severe respiratory clinical signs than birds infected with CalEnt at all time points (4, 6, 10 and 14 dpi) ( $P<0.05$ , Figure 2). Within the M41-infected groups, birds inoculated oculonasally presented higher respiratory sign scores than birds inoculated via crop gavage at 4 dpi. In contrast, birds infected with M41 via crop presented with the highest respiratory sign score at 14 dpi ( $P<0.05$ , Figure 2).



**Figure 2.** Respiratory sign scores of commercial broilers challenged with either IBV M41 or CalEnt oculonasally or via crop gavage. Different superscripts represent statistical significance ( $P<0.05$ ).

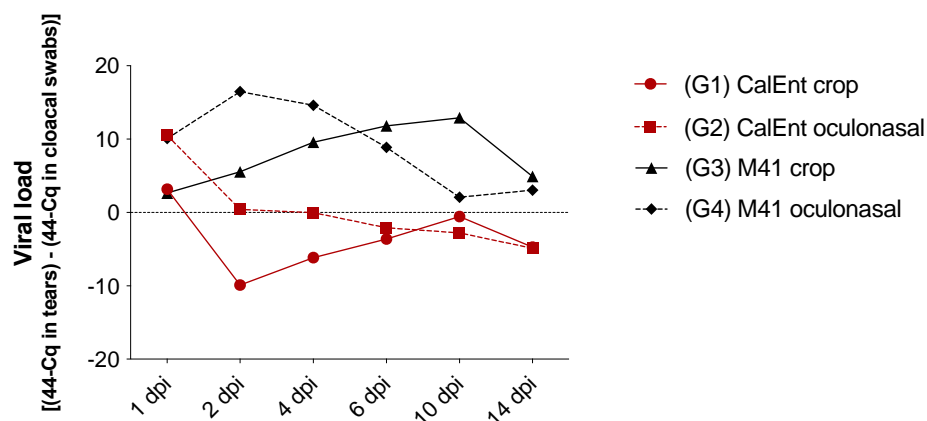
Vent staining was observed more frequently in CalEnt-infected birds throughout the experiment, although no statistical differences were observed between IBV-infected birds (Figure 3). Birds challenged with CalEnt oculonasally appeared to present diarrhea more frequently than other groups at 6 dpi ( $P<0.05$ , Figure 3). The unchallenged control birds also presented stained vents at 6, 10 and 14 dpi; however, the frequency was significantly lower than in IBV-challenged groups ( $P<0.05$ , Figure 3).



**Figure 3.** Percentage of commercial broilers presenting vent staining after a challenge with either IBV M41 or CalEnt oculonasally or via crop gavage. Different superscripts represent statistical significance ( $P < 0.05$ ).

### 3.4. Viral shedding

In general, the viral load in tears was higher in M41-challenged birds than in those challenged with CalEnt. At 1, 2 and 4 dpi, the highest viral load were seen in birds challenged with M41 oculonasally; at 6 dpi and onward, the viral load in tears was higher in birds challenged with M41 via crop ( $P < 0.05$ ). With the exception of swabs collected at 1 dpi, the viral loads in cloacal swabs were overall higher in CalEnt-infected chickens, although only statistically significant at 2 and 10 dpi ( $P < 0.05$ ). Figure 4 shows the difference between the average viral load in tears and cloacal swabs. Starting at 2 dpi, the viral load difference is above zero in M41-infected birds, indicating higher viral shedding in the upper respiratory tract. In contrast, the viral load difference in chickens challenged with CalEnt is below zero, denoting higher viral elimination in feces in these birds. Statistical differences between viral loads are represented in Supplementary Table S4.



**Figure 4.** Difference between viral load in tears and viral load in cloacal swabs collected from commercial broilers challenged with either IBV M41 or CalEnt oculonasally or via crop gavage. Numbers above zero represent a higher viral load in tears. Numbers below zero represent a higher viral load in cloacal swabs. Group 5 (unchallenged controls) is not represented because no IBV amplification was observed in any sample at any timepoint.

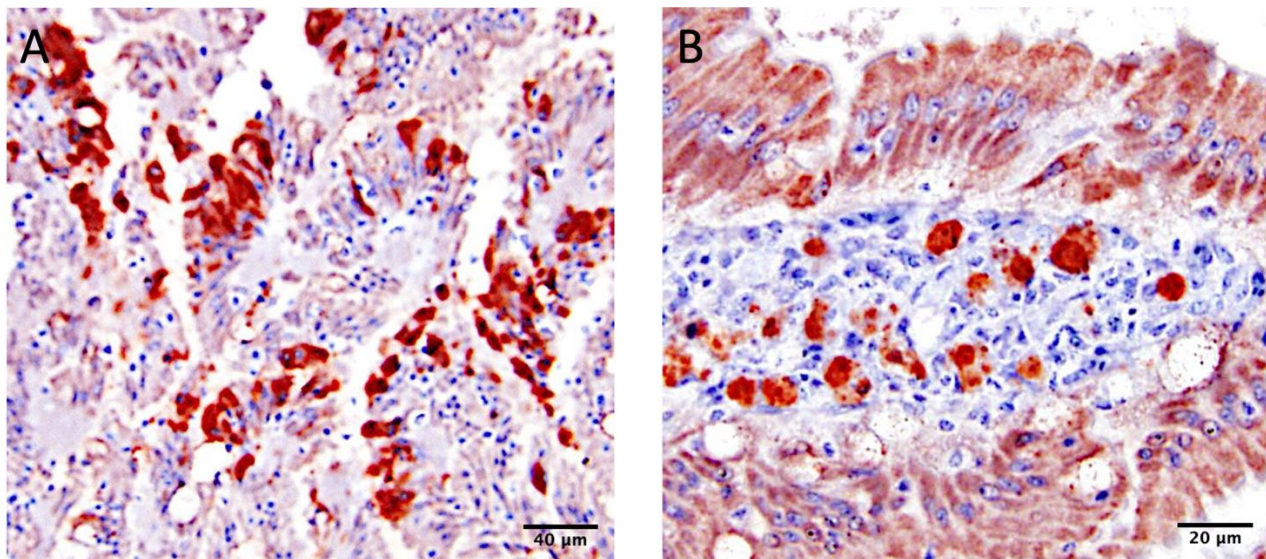
### 3.5. Histopathology and immunohistochemistry

At 4 dpi, CalEnt-challenged birds presented with mild to moderate lymphoplasmacytic infiltration of the tracheal mucosa, while the lesions in M41-infected birds were scored as moderate to severe. The IHC showed a small amount of virus antigen present in the tracheas of CalEnt-infected chickens, while the amount of IBV antigen in M41-infected birds was moderate. The presence of tracheal lesions was irrespective of the



inoculation route. IBV viral antigen was not detected by IHC in tracheas of any birds at 14 dpi.

Intestinal lesions were observed in all groups, including the unchallenged controls (group 5). A few birds in each group had small numbers of coccidia. At 4 dpi, mild to moderate IBV IHC staining was present in the cytoplasm of enterocytes at the tips of the villi of the duodenum and ileum from birds challenged with CalEnt (Figure 5A). In contrast, none to small amounts of viral antigen was observed in the intestines of M41-challenged birds at 4 dpi. At 14 dpi, mild to moderate virus staining was detected in the cytoplasm of lymphocytes present in the intestinal lamina propria of birds challenged with CalEnt (Figure 5B). No IHC staining was observed in M41-infected chickens or unchallenged controls.



**Figure 5.** Infectious bronchitis virus antigen detection in intestinal sections of broilers challenged with CalEnt by immunohistochemistry (IHC). (A) IBV IHC staining in the cytoplasm of enterocytes from CalEnt-infected chickens at 4 dpi via oculonasal route. Scale bar = 40 µm. (B) IBV IHC staining in the cytoplasm of lymphocytes of the intestinal lamina propria from CalEnt-infected chickens at 14 dpi via oculonasal route. Scale bar = 20 µm.

#### 4. Discussion

Although IBV is typically known for inducing respiratory and urogenital disease, few IBV strains have been described as being mainly enterotropic [9-12]. The IBV CalEnt strain has been associated with a case of runting-stunting in 14-day-old brown/red broiler chicks. When CalEnt was replicated in SPF embryonated eggs, the virus was retrieved from embryo intestines, but not from allantoic fluid or the chorioallantoic membrane. After an *in vivo* challenge experiment with IBV CalEnt, the enteritis and stunting was successfully reproduced in a laboratory setting, suggesting an enteric tropism [14]. Despite all the efforts in characterizing the pathobiology of IBV strains with supposed enteric tropism, information on the molecular aspects of such strains are scarce. Here, we provide a comprehensive genomic characterization of CalEnt and a glimpse into the pathological process of this particular IBV strain in commercial broiler chickens.

The complete genomic sequence of CalEnt showed homologies higher than 94% to Cal 99, Cal 56b, M41 and Ark DPI (Table S1). Despite this relatively high whole-genome identity to known and established IBV strains, CalEnt is still rather unique in nucleotide composition, and branches separately from other IBV strains phylogenetically (Figure 1A). When the phylogenetic and identity analyses were restricted to the most variable part of the viral genome – the S gene – the similarity between CalEnt and Cal 99 becomes more evident (Figures 1B and 1C). When narrowing down the analysis to the hypervariable region of the S gene, a striking identity of 94.48% between CalEnt and Cal 99 was observed (Table S3), which is supported by their solid clustering in Figure 1C. It is noteworthy that,

in this instance, the S1 hypervariable region was less variable than the remainder of the S gene. The S gene is the main determinant of tissue tropism and host specificity [25], and therefore is the most relevant gene for genotyping of IBV strains. However, considering other genes, CalEnt also showed very high identity to other IBV isolates commonly used in vaccines in California, such as Mass, Conn, and Ark strains (Table 2). These results suggest that CalEnt is an IBV variant that arose from continued virus mutations in the presence of vaccine strains. The emergence of IBV variants such as CalEnt in 2012 [14] and Cal 99 in 1999 [26,27] likely resulted from vaccine mishandling in the field, either by poor application techniques or by usage of vaccines at a lower dosage than the recommended by the manufacturer. Vaccination issues may lead to rolling reactions and back-passaging of vaccine viruses within the flock, providing opportunities for mutations and recombination events that generate novel variants over time that might circumvent vaccine immunity [28].

Clinically, the commercial broilers challenged with either M41 or CalEnt in our experiment presented with respiratory signs and lesions, regardless of the route of inoculation or IBV strain, although respiratory disease was more severe in M41-infected birds than those challenged with CalEnt (Figure 2). A similar effect was observed in other studies investigating IBV strains isolated from the intestines, in which birds initially present with respiratory signs [9,12]. In addition, the viral load in tears was overall higher in M41-infected birds (Figure 4, Table S4), suggesting the viral shedding of M41 in the upper respiratory tract is higher than that of CalEnt. In contrast, vent staining was slightly more frequent in CalEnt-infected birds (Figure 3). Furthermore, the intestinal microscopic lesions and IBV IHC detection was more severe and the viral shedding in feces was overall higher with the CalEnt challenge compared with M41 (Figure 4, Table S4). However, the M41 challenge dose was 10 times higher than that of CalEnt due to difficulties in amplification of the wild-type CalEnt strain in SPF eggs. Nevertheless, the high frequency of diarrhea and the high shedding in feces are still noteworthy; perhaps these observations would have been more noticeable if the viral challenge was higher. An interesting observation is that, at 14 dpi, all IBV-infected groups were still shedding the virus via feces, which supports the existing knowledge that IBV persists in cecal tonsils for longer periods, making this tissue a good specimen for the detection and isolation of IBV [2].

Commercial broilers vaccinated against coccidia and *Salmonella* at the hatchery were used in this study, and some baseline intestinal damage might have been caused by these live vaccines. Intestinal health in commercial poultry is extremely complex and involves numerous factors, including attenuated microorganisms provided in vaccines, the microbiota, co-infections with other pathogens and the nutritional contents of the feed [29]. In addition, a previous study using CalEnt failed to demonstrate the binding of the viral S1 protein to the tracheal or intestinal epithelia, suggesting that CalEnt might have lower affinity to the host cell than other IBV strains *in vitro* [30]. It is possible other factors present in the enteric milieu of commercial chickens (i.e., microbiome and tissue-specific proteases) facilitate the CalEnt binding to epithelial cells. By using commercial broiler chickens, our goal was to mimic a realistic poultry farm setting to understand the performance of the two IBV strains in the presence of other microorganisms. Despite the confounding lesions possibly caused by the coccidia and *Salmonella* vaccines, the unchallenged control birds presented with less severe intestinal lesions compared to IBV-infected birds, and no IBV IHC staining was observed. The microscopic injuries observed in the CalEnt-infected birds appeared slightly more severe than those present in M41-infected birds, and the IBV IHC staining was more evident, supporting the enteric tropism of CalEnt as previously reported [14].

Regarding the inoculation route, the crop gavage inoculation was performed to investigate if fecal-oral infection would have a different impact on CalEnt pathogenesis as opposed to the known airborne transmission of IBV. In general, it appeared that the crop gavage inoculation led to a late onset of clinical disease that persisted for a prolonged period compared with the oculonasal inoculation. It is possible that a portion of the



inoculum was inactivated in the proventriculus or ventriculus of challenged chickens, reducing the viral load and consequently delaying the clinical signs and viral shedding.

## 5. Conclusions

This study corroborates the evidence that CalEnt has a tropism for the intestinal tract, which is unusual for IBV. Its comprehensive pathogenesis and pathobiology in SPF chickens, its influence in intestinal health and the underlying molecular mechanisms of this preferred replication site remains to be determined.

**Supplementary Materials:** The following are available online at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Table S1: Percent nucleotide identity of infectious bronchitis virus sequences using the complete viral genomes aligned using MAFFT [17], Table S2: Percent nucleotide identity of infectious bronchitis virus sequences using full spike gene sequences aligned using MAFFT [17], Table S3: Percent nucleotide identity of infectious bronchitis virus sequences using a 751-bp fragment of spike gene bearing the S1 hypervariable region; sequences aligned using MAFFT [17], Table S4: Mean viral loads in tears and cloacal swabs from birds challenged with either infectious bronchitis CalEnt or M41 strains via crop gavage or oculonasally (ON) at day of infection at 1, 2, 4, 6, 10 and 14 days post-infection (dpi). Values are represented as 44 (number of cycles in the RT-qPCR) minus the C<sub>q</sub>. Different superscripts represent statistical significance ( $P < 0.05$ ) within a column and within a specimen. SD = standard deviation.

**Author Contributions:** Conceptualization, R.G. and R.H.; methodology, R.G. and R.H.; bioinformatics, A.D.S. C.K., H.Z.; formal analysis, A.D.S., R.H., R.G.; investigation, R.H., S.N., R.G.; histopathology, H.L.S.; funding acquisition, R.G.; data curation, R.H. A.D.S.; writing—original draft preparation, R.H., R.G., A.D.S.; writing—review and editing, R.H., R.G., A.D.S., H.L.S.; supervision, R.G.; project administration, R.G. All authors have read and agreed to the published version of the manuscript.

**Funding:** Please add: This research was funded by the Center for Food Animal Health (CFAH) at the University of California, Davis with NIFA multistate project funding (NC1180).

**Acknowledgments:** We would like to acknowledge the poultry medicine laboratory members at the University of California, Davis for their diligent work and support.

**Conflicts of Interest:** The authors declare no conflict of interest.

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