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Isolation, identification, molecular and pathogenicity characteristics of an infectious laryngotracheitis virus from Hubei province, China

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ABSTRACT Multiple outbreaks of avian infectious laryngotracheitis (**ILT**) in chickens, both domestically and internationally, have been directly correlate to widespread vaccine use in affected countries and regions. Phylogenetic and recombination event analyses have demonstrated that avian infectious laryngotracheitis virus (**ILTV**) field strains are progressively evolving toward the chicken embryo-origin (**CEO**) vaccine strain. Even with standardized biosecurity measures and effective prevention and control strategies implemented on large-scale farms, continuous ILT outbreaks result in significant economic losses to the poultry industry worldwide. These outbreaks undoubtedly hinder efforts to control and eradicate ILTV in the future. In this study, an ILTV isolate was successfully obtained by laboratory PCR detection and virus isolation from chickens that exhibited dyspnea and depression on a broiler farm in Hubei Province, China. The isolated strain exhibited robust propagation on chorioallantoic membranes of embryonated eggs, but failed to establish effective infection in chicken hepatocellular carcinoma (**LMH**) cells. Phylogenetic analysis revealed a unique T441P point mutation in the gJ protein of the isolate. Animal experiments confirmed the virulence of this strain, as it induced mortality in 6-wk-old chickens. This study expands current understanding of the epidemiology, genetic variations, and pathogenicity of ILTV isolates circulating domestically, contributing to the elucidate of ILTV molecular basis of pathogenicity and development of vaccine.

Key words: infectious laryngotracheitis virus, gJ mutation, phylogenetic analysis, Hubei

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INTRODUCTION

Infectious laryngotracheitis (**ILT**) is an acute and highly contagious respiratory infectious disease in chickens caused by infectious laryngotracheitis virus (**ILTV**), also known as gallid herpesvirus type I. Depending on virulence of the strain, ILT exhibits diverse clinical symptoms. Moderate infections manifest as conjunctivitis, tracheitis, and rhinitis, while acute infections often present with dyspnea, tracheal mucosal hemorrhage, and even hemoptysis (Hughes et al., 1991a). As a member of the Herpesviridae family, ILTV invades the trigeminal ganglia and establishes latent infections as important survival mechanisms (Hughes et al., 1991b). Chickens are the primary natural hosts for ILTV, with birds of all ages and breeds susceptible, although those over 4-wk-old are most affected (Portz et al., 2008). Ducks and pigeons, however, are insensitive to ILTV. The disease rapidly spreads with high morbidity but variable mortality (Kirkpatrick et al., 2006). Survivors of moderate infections exhibit growth retardation and decreased egg production. Consequently, ILT is prevalent worldwide, especially in intensive poultry farming regions such as Europe, the United States, Australia and China. These outbreaks unquestionably lead to tremendous economic losses and pose a serious threat to the stability and development of the poultry industry (Garcia and Zavala, 2019; Mossad et al., 2022; Ponnusamy et al., 2022).

The ILTV genome is a linear double-stranded DNA of approximately 155 kb in size, consisting of unique long

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(**UL**) regions and unique short (**US**) regions, flanked by inverted repeat regions known as internal repeat sequences (**IRs**) at the N-terminal, and terminal repeat sequence (**TRs**) at the C-terminal (Fuchs et al., 2007). The envelope is studded with glycoprotein spikes. The viral genome contains nearly 80 open reading frames (**ORFs**) that can be classified into immediate early, early and late genes based on kinetics of herpesvirus gene expression (Honess and Roizman, 1974; Guo et al., 1993). Those encoded proteins of ILTV are involved in nucleotide metabolism, DNA replication, DNA repair and post-translational modifications, etc. (Devlin et al., 2006; Helferich et al., 2007; Mundt et al., 2011; Pavlova et al., 2013). Mature virions are composed of, from inside out, a viral core, capsid, tegument and envelope. Additionally, the genome encodes 11 conserved glycoproteins. Among them, the thymidine kinase (**TK**) related to virulence and also serves as a classic site for integration of heterogenous genes (Keeler et al., 1991). The gB, gC, and gD associated with viral attachment and entry, and can elicit host humoral and cellular immunity (Kingsley et al., 1994; Poulsen and Keeler, 1997). The gG plays an important role in viral immune evasion (Devlin et al., 2006). While the aforementioned proteins have been thoroughly characterized, the functional and mechanistic characterization of most other ILTV proteins remains incomplete. Since its initial isolation and identification in Guizhou, China in the 1960s, ILT outbreaks have rapidly prevalent throughout several Chinese provinces, including Hebei, Henan, Jiangsu, and Shandong (Wu et al., 2022). As a result, ILT has emerged as one of the predominant epidemic diseases afflicting large-scale poultry farms in China. Attenuated vaccines based on chicken embryo-origin (**CEO**) and tissue culture-origin (**TCO**) strains, as well as recombinant vector vaccines, have provided potent means of ILTV control (Coppo et al., 2013). However, the increasing frequency of ILT outbreaks and the emergence of vaccine-derived strains have inevitably posed new challenges for ILTV prevention and control. Currently, our understanding of the epidemiology, genomic variations among circulating strains, and viral pathogenicity and biological characteristics of ILTV in China remains limited.

In this study, a suspected ILTV was isolated from diseased chickens on a broiler farm in Hubei Province, China. PCR identification excluded common avian respiratory pathogens and confirmed the isolate as ILTV, designated as HBHG2301. The HBHG2301 stably propagates in chorioallantoic membranes (**CAM**) of specific pathogen free (**SPF**) embryos, and continuous passage enhanced its virulence in embryos. However, HBHG2301 failed to establish effective infection in chicken leghorn male hepatocellular (**LMH**) cells. Phylogenetic analysis of the US5 gene (encoding gJ protein) revealed ~99% nucleotide identity among HBHG2301, the rarely used CEO vaccine strain Serva, and a Chinese field isolate LJS09 (2012) (Kong et al., 2013), with a single nucleotide mutation at position 1,321 that resulted in a unique T441P substitution in HBHG2301 gJ, at a site conserved among all reported ILTV gJ sequences. Animal experiments demonstrated high virulence of HBHG2301, inducing sudden death in 6-wk-old SPF chickens. Interestingly, while ILTV was detected in organs and throat swabs of low-dose infected chickens, and pathological examinations revealed viral-induced laryngeal and tracheal swelling and hemorrhage, infected chickens exhibited no overt clinical signs such as conjunctivitis or respiratory distress. Further analyses are warranted to examine potential associations between the biological characteristics and pathogenicity of HBHG2301 and its unique T441P mutation in gJ. In summary, this study isolated a novel variant ILTV field strain, expanding current knowledge of ILTV evolution during natural infection, and providing an insight for improved ILT control and vaccine development.

MATERIALS AND METHODS

Samples, Cells, and Virus Isolation

The ILTV HBHG2301 was isolated from diseased chickens on a broiler farm in Hubei Province, China in 2022, and identified by PCR using primers for common avian respiratory pathogens (Table 1). This farm had not administered any ITLV live vaccines. For virus isolation, briefly, lung, larynx and tracheal tissues were collected from 2- to 3-mo-old chickens in the herd displayed typical symptoms of respiratory distress and conjunctivitis. The harvested samples were homogenized in PBS (containing 5% penicillin and streptomycin) at a 1:10 mass/volume ratio, frozen and thawed 3 times at −80°C, then centrifuged at 12,000 rpm/min for 30 min, and finally the supernatant filtered through a 0.22 μm filter (Millipore, Germany, SLGV033RB).

The filtrate was then isolated and passaged in 10-day-old SPF Chick embryos (Beijing Boehringer Ingelheim Viton Biotechnology Co., Ltd., China) through CAM route. The CAM and allantoic fluid were harvested 5 days postinoculation (Ebrahimi et al., 2021). After grinding and filtering with 0.22 μm filter, the homogenates were finally stored at −80°C. The LMH cells were stored in our lab and cultured with DMEM

Table 1. Primers used for clinical samples detection.

Pathogens	Forward primer 5′−3′	Reverse primer 5′−3′
Avian infectious bronchitis virus	CGGAATTCAGTTTCCTAAGAACGGTTGGAA	CCCAAGCTTCTCTACACGCACACATTTAT
H5 Avian influenza virus	AGTGAATTGGAATATGGTAACTG	AACTGAGTGTTCATTTTGTCAAT
H7 Avian influenza virus	AATGCACARGGAGGAGGAACY	TGAYGCCCCGAAGCTAAACCA
H9 Avian influenza virus	TCAACAAACTCCACCGAAACTGT	TCCCGTAAGAACATGTCCATACCA
New castle disease virus	ATGGGCYCCAGAYCTTCTAC	CTGCCACTGCTAGTTGTGATAATCC
Infectious laryngotracheitis virus	CTCTTCCTCCTCTTCCTCAT	GTTACTGACTGAACCGACCC

(ThermoFisher, 12100061) containing 10% fetal bovine serum (SORFA, China, SX1101).

gJ Sequencing and Sequence Analysis

Viral genome DNA was extracted from samples using the TIANamp Genomic DNA kit (TIAGEN, China, DP304) according to the manufacturer instruction. The gJ sequence was amplified using the primers (designed using Primer 6) ILTV-gJ-F 5′-CATCAGGTGTGGTTTGCGTGTCT-3′ and ILTV-gJ-R5′-GCGAAGGAAGAAAGCAGTACGATAGT-3′. Two more middle primers were used for sequencing (Tsingke Biotechnology, China), ITLV-H01 5′-TTTGACGGTGGAAACGCAAG-3′ and ITLV-B02 5′-GCAGTACATTGGGGAGTGCT-3′. To ensure the authenticity of the final sequences, 3 independent samples were selected for amplification, sequencing, and alignment during each sequencing and assembly. The HBHG2301 gJ sequence was assembled by SnapGene and submitted to GeneBank (Accession number OR400418). The data were to be held confidential until Feb 13, 2024. Phylogenetic trees were constructed by MEGA 11 using Neighbor-Joining method (Bootstraps = 1,000).

Virus Multiplication

The method for viral multiplication on chicken embryos was as follows: After filtering the homogenized samples (ILTV PCR positive), the supernatant was inoculated through the CAM pathway into 10-day-old SPF chicken embryos with 200 μL per embryo, including a negative control group. The embryos were then incubated at 37°C incubator for 5 d. Embryos that died within 24 h were discarded. After 5 d of infection, the allantoic fluid and CAM were collected and homogenized, then centrifuged at 12,000 rpm/min for 30 min and finally the supernatant filtered through a 0.22 μm filter. Afterward, the filtered and clarified supernatant was used for further passage. The EID_{50} of the virus was determined using SPF chicken embryos (the Reed −Muench method; Reed and Muench, 1938). The samples were serially diluted by 10-fold dilution and inoculated through CAM with 8 embryos per dilution. The embryos were then observed for 5 d, and the presence of white pock-like necrotic plaque on the CAM was considered as ILTV positive.

The multiplication of ILTV in LMH cells was conducted as follows: LMH monolayers were inoculated with ILTV and incubated at 4°C for 2 h. The supernatant was discarded and replaced with DMEM containing 1% serum. When obvious CPE was observed or cultures reached 120 h, infected cells and medium were collected, freeze-thawed, centrifuged, and the supernatant were finally harvested. The viral titer of the supernatants was determined via EID_{50} and quantitative fluorescent PCR (Callison et al., 2007; Vagnozzi et al., 2012).

Animal Experiment

A total of 49 SPF chickens (6-wk-old) were randomly divided into 4 groups, 3 groups were inoculated 100 μL of CEO HBHG2301 intratracheally at 3 different doses ($10^{3.43}$EID50, $10^{2.43}$EID50 and$10^{1.43}$EID50, respectively). The control group was given 100 μL of PBS via the same route. Chickens were monitored continuously for 14 d in negative pressure isolators. Throat swabs were collected from each animal on d 3, 5, 7, and 9 postinfection for PCR detection. Finally on d 14 postinfection, chickens were humanely euthanized for necropsy examination and documentation of gross lesions, followed by PCR testing of susceptible organs. The experiment was conducted in accordance with the principles and specific guidelines presented in Guide for the Care and Use of Agricultural Animals in Research and Teaching, 4th ed., 2020.

Data Analysis

All experiments were performed independently 3 times and the data in tables were presented as mean and standard deviation (**SD**).

RESULTS

Isolation and Identification of ILTV From Broiler Chickens

In 2022, a putative infectious laryngotracheitis virus was isolated and purified from symptomatic broiler chickens from a poultry farm in Hubei Province, China. This farm had not administered any ITLV live vaccines. Polymerase chain reaction (**PCR**) testing using primers for common avian respiratory pathogens (see details in Materials and Methods) yielded negative results except ILTV. Inoculation of the PCR-positives tissue homogenate via the CAM route into 10-day-old SPF chicken embryos resulted in considerable thickening of the CAM and white pock-like necrotic plaques (Figure 1). Serial passage in chicken embryos showed an improvement of viral titer from $10^{2.67}EID_{50}/200$ μL to $10^{4.43}EID_{50}/200$ μL (Table 2). The isolation was designated as HBHG2301.

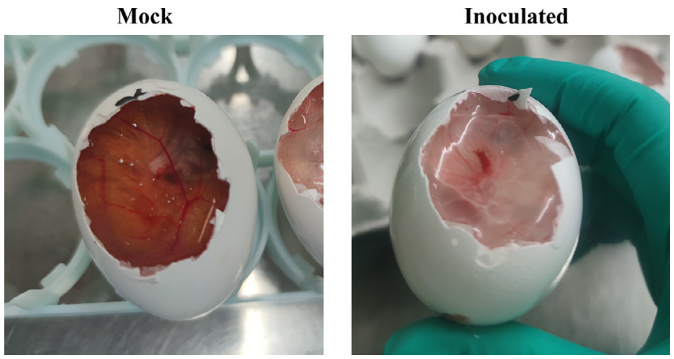

Figure 1. Pathological changes in CAM on the fifth day postinoculation.

Table 2. Serial passages of ILTV in SPF chicken embryos.

Passage	$Log_{10}EID_{50}/200\ \mu L$
1	2.59 ± 0.09
3	3.13 ± 0.12
5	4.21 ± 0.22

Molecular Characteristics and Phylogenesis Analysis of HBHG2301 US5 Gene

As a member of the herpesvirus family, the genome of ILTV is also highly conserved. Therefore, strain differentiation and subtyping of field isolates, as well as differentiation from vaccine strains, is difficult to achieve by PCR given the large size of the genome. Hence, the US5 gene, which displays the highest degree of variability in the ILTV whole genome, was selected for further analysis. Firstly, sequence analysis of the US5 gene amplified from the clinical sample demonstrated complete identity when compared to sequences obtained from the serially passaged isolates in vitro, with no amino acid residue mutations. Further sequencing, alignment and analysis were performed among HBHG2301 US5 nucleotide sequence and published vaccine strains (domestic or foreign strain), as well as circulating strains in China, the U.S, Australia and surrounding countries (Table 3). As shown in Table 4, The US5 gene sequence of HBHG2301 (OR400418) displayed high homology compared to the domestic vaccine strain Serva (Chicken embryo-origin), wild strain LJS09 (2012), American vaccine strain LT Blen and Laryngo Vac (Chicken embryo-origin), American isolate 14939 (2017), and Australian isolate CL9 (2011), with the exception of an adenine to thymine mutation at nucleotide position 1,321, resulting in a unique T441P amino acid mutation of glycoprotein J in the HBHG2301 strain. Apart from that, compared to the domestic vaccine strain K317, the HBHG2301 strain obtained 4 mutations (E88G, L264M, K138M, T441P) that located at the N-terminus of gJ protein.

To further investigate the phylogenesis relationships of ILTV US5 genes, a phylogenetic tree was generated (Figure 2). The results showed that the HBHG2301 strain, marked in red, was clustered with those CEO-origin vaccine strains and was more closely related to the Serva vaccine strain than the K317 strain. Additionally, HBHG2301 and the virulent Chinese isolate WG did not cluster on the same branch.

Table 3. Detailed information of the ILTV genome that used in this study.

Strain name	GeneBank accession no.	Location	Vaccine/Filed
HBHG2301	OR400418(gJ)	China	Filed
Serva	HQ630064	Australia	Vaccine
K317	JX458824	China	Vaccine
TCO-IVAX	JN580312	United States	Vaccine
SA2	JN596962	Australia	Vaccine
A20	JN596963	Australia	Vaccine
LT Blen	JQ083493	United States	Vaccine
Laryngo Vac	JQ083494	United States	Vaccine
CEO-TRVX	JN580313	United States	Vaccine
WG	JX458823	China	Filed
LJS09	JX458822	China	Filed
1874C5	JN542533	United States	Filed
81658	JN542535	United States	Filed
63140	JN542536	United States	Filed
CL9	JN804827	Australia	Filed
V1-99	JX646898	Australia	Filed
CSW-1	JX646899	Australia	Filed
14939	MF417811	United States	Filed
KO0206	MH937564	Korea	Filed
KO030678	MH937565	Korea	Filed
KO440798	MH937566	Korea	Filed

Cytopathogenicity of HBHG2301 in LMH Cells

The gJ protein encoded by US5 gene is an important envelope protein of ILTV that assembles on the surface of viral particles and is involved in viral exocytosis, but with no responsibilities for cell-to-cell spreading (Mundt et al., 2011). Interestingly, the isolated strain HBHG2301 in this study possessed a unique T441P mutation in gJ that has never been reported in other ILTV strains (Table 4). Therefore, we first examined the infection and propagation of HBHG2301 on LMH cells. CEO LITV (passage 3 and 5) were serially passaged on LMH cells for 5 more times. Interestingly, only infection with CEO virus could induce typical cytopathic effect in LMH cells, with significant cell swelling and nuclear fusion observed 96 h postinfection compared to controls (Figure 3). ILTV after continuous passage in LMH cells failed to generate distinct cytopathic effects as described above. Correspondingly, quantitative PCR detection of viral titers in each passage revealed that while HBHG2301 could achieve limited propagation in LMH cells, viral titers decreased with later passages, with an approximately 100-fold reduction in P5 compared to P1 (Table 5). Additionally, the pathogenicity of cell-culture passaged ILTV in chicken embryos was gradually abolished, with P4 and P5 showing almost complete loss of virulence in embryos.

Pathogenicity of HBHG2301 in Natural Reservoir

To further characterize the pathogenicity of HBHG2301, an infection model was established in 6-wk-old SPF chickens. A total of 49 SPF chickens were randomly divided into 4 groups. As shown in Table 6, chickens were monitored continuously for 14 d in negative pressure isolators. In the highest dose group ($10^{3.43}EID_{50}$ per chicken), 3 of 15 died suddenly on d 3 postinfection (Figure 4A). PCR testing of throat swabs from these 3 chickens were positive. Necropsy revealed swollen larynxes congested with copious mucus exudates and blood clots within the tracheal lumen, as well as hyperemic and edematous tracheal mucosa. PCR testing of the trachea, larynx, and lungs were all positive, consistent with acute ILTV infection. Throat swabs from the 3 challenged groups exhibited near 100% (11/12 in $10^{3.43}EID_{50}$ group, 12/12 in $10^{2.43}EID_{50}$ group and 12/

Table 4. Variation analysis of ILTV US5 gene at indicated position, from US5 ATG.

Strain	23[1]	207	221	231	263	312	484	488	660	736	765	770	777	790	878	903	933	943	1009	1018	1075	1276	1321[3]	1388	1427	1481	1505	1559
HBHG2301	G[2]	C	T	C	G	C	C	G	G	A	C	C	T	A	T	G	G	T	C	G	A	A	C	C	C	C	C	A
Serva																							A					
K317					A								C	T				A					A					
TCO-IVAX							T								C								A					
SA2	A								T		T					C			G			G	A			T	A	
A20	A								T		T					C			G			G	A			T	A	
LT Blen																							A					
Laryngo Vac																							A					
CEO-TRVX													C										A					
WG									T		T					C			G			G	A				A	C
LJS09																							A					
1874C5											T												A					
81658							T								C								A					
63140																				A			A					
CL9																							A					
V1-99	A	T		T							T	T				C	A				C		A	T		T		
CSW-1	A	T	C	T				A			T	T				C	A				C		A	T		T		
14939																							A					
KO0206																							A					
KO030678	A					T				G	T					C					C	G	A		G	T		
KO440798													C										A					

Strain	1576	1632	1641	1665	1750	1799	1860	1926	1927	1940	1952	1960-1989[4]	2001	2019	2080	2143	2156	2160	2163	2659	2692	2805	2856
HBHG2301	C	C	G	T	C	A	G	A	C	C	G	Del	T	A	G	G	C	T	C	C	G	C	A
Serva												Del											
K317												Del											
TCO-IVAX												Del											
SA2						G				T		+30		G				C		T			G
A20						G				T		+30	G	G				C		T			G
LT Blen												Del											
Laryngo Vac												Del											
CEO-TRVX												Del											
WG						G		G	T	T		+30		G				C		T			G
LJS09												Del											
1874C5												Del			A								
81658												Del											
63140												Del											
CL9												Del											
V1-99		T	T	C	T		A					Del		G		A	A		T	T		T	G
CSW-1		T	T	C	T		A					+30		G		A	A		T	T		T	G
14939												Del											
KO0206												Del									A		
KO030678	T										A	Del		G									G
KO440798												Del											

[1]Nucleotide sequence numbered from HBHG2301 ATG.
[2]The nucleotide sequence of strain HBHG2301 is shown below at the indicated positions. Empty text boxes for the other strains signify identity to the HBHG2301 sequence at those sites.
[3]A unique mutation of HBHG2301 at A1321C from all other strains in GeneBank (26/7/2023).
[4]Del, 30 bp deletion; +30 bp, CCGGAAATTACTCAGACTCCGAGTACGGTA insertion.

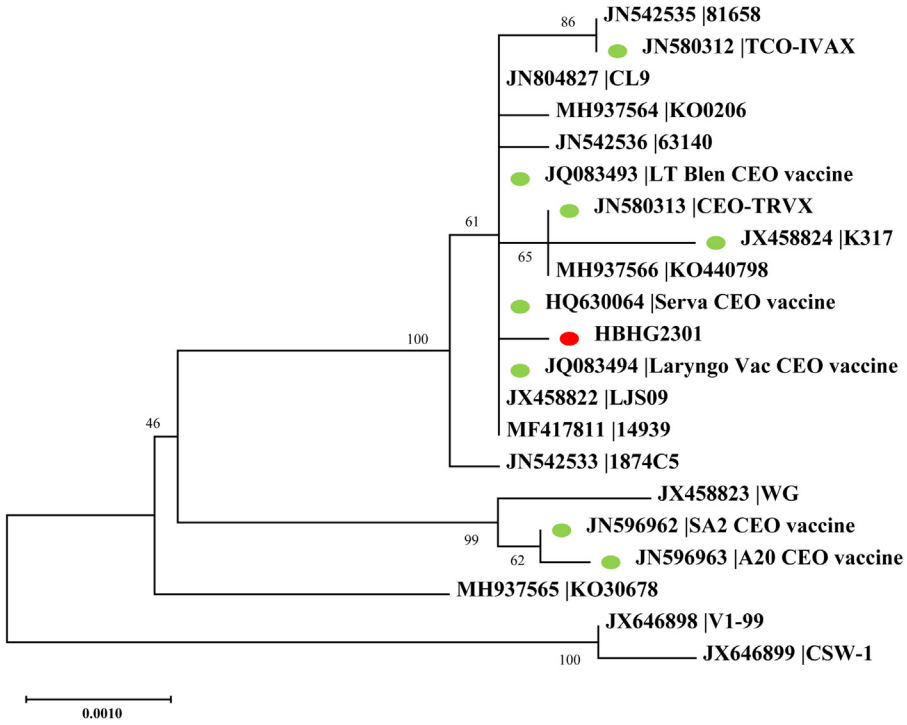

Figure 2. . Phylogenetic tree constructed based on gJ sequences. Red circle, HBHG isolated is this study. Green circle, vaccine strains.

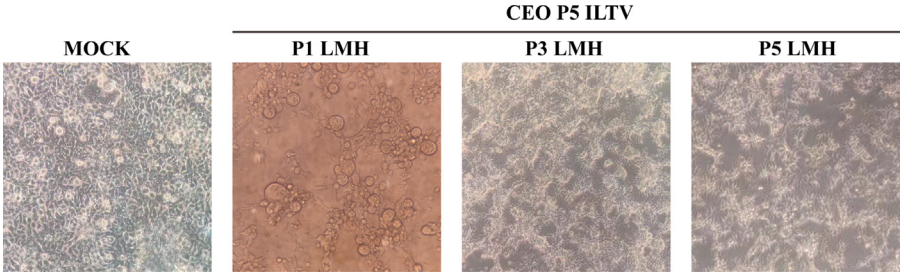

Figure 3. Serial passaging of CEO HBHG2301 in LMH cell. P1, passage 1. P3, passage 3. P5, passage 5; 10× objective lens.

12 in $10^{1.43}$EID$_{50}$) positivity over the first 5 d, followed by a noticeable decline to 20 to 50% (3/12 in $10^{3.43}$EID$_{50}$ group, 6/12 in $10^{2.43}$EID$_{50}$ group and 6/12 in $10^{1.43}$EID$_{50}$) in a week. Some samples tested negative on d 7 but positive again on d 9, likely due to variability in PCR sensitivity. Necropsy examination on d 14 postinfection revealed the highest nucleic acid positivity rates across challenged groups were observed in the trachea, at 73.33% (11/15 in $10^{3.43}$EID$_{50}$ group), 25% (3/12 in $10^{2.43}$EID$_{50}$ group), and 16.67% (2/12 in $10^{1.43}$EID$_{50}$). Unexpectedly, lung tissues from the highest dose group showed 80% (12/15) positivity, indicating HBHG2301 not only infects but actively replicates within the lungs. Further necropsy examination revealed varying degrees of hyperemia and hemorrhage within the tracheal mucosa of all 3 infected groups compared to controls (Figure 4B). Petechial hemorrhages were observed in the larynx, with decreasing laryngeal edema and hemorrhage density associated with lower infection doses. Infected lung tissues exhibited localized hyperemia compared to controls.

Table 5. The HBHG2301 virus proliferation on LMH cells.

Virus	Passage on LMH cells	ΔCT values	$\log_{10}$EID$_{50}$/200 μL
P3 Chicken embryo-origin	P1	14.42 ± 0.08	1.61 ± 0.10
	P2	20.93 ± 0.02	0.00
	P3	19.67 ± 0.03	1.18 ± 0.17
	P4	20.37 ± 0.09	0.00
	P5	21.70 ± 0.03	0.00
P5 Chicken embryo-origin	P1	11.23 ± 0.03	2.00 ± 0.10
	P2	12.97 ± 0.04	1.49 ± 0.19
	P3	14.34 ± 0.03	1.34 ± 0.14
	P4	19.06 ± 0.02	0.00
	P5	20.14 ± 0.03	0.00

DISCUSSION

The rational utilization of ILTV vaccines and the implementation of scientific control measures against ILT epidemics are essential factors for preventing outbreaks and minimizing losses in poultry farming. Therefore, investigation the pathogenesis and epidemiology of ILTV is particularly critical. In this study, an ILTV isolate HBHG2301 was successfully recovered from tissue homogenate. Serial passages in CAM demonstrated markedly enhanced virulence in chicken embryos (Table 2). However, contradictory results were observed

Table 6. PCR test of the swabs and samples in HBHG2301 infected and mock groups.

		PCR of throat swabs				PCR of viscera and organs at 14-day postinfection		
Group	No.	Day 3	Day 5	Day 7	Day 9	Throat	Trachea	Lung
Positive rate		100.00%	91.67%	25.00%	16.67%	46.67%	73.33%	80.00%
$10^{3.43}EID_{50}$ number = 15	H194	+	+	-	-	-	+	+
	H195	+	+	-	-	-	+	+
	H196	+	/	/	/	+	+	+
	H197	+	-	-	-	+	+	+
	H198	+	+	-	-	+	+	+
	H199	+	/	/	/	+	+	+
	H254	+	+	-	-	-	+	+
	H255	+	+	-	-	+	-	+
	H256	+	+	-	-	-	+	+
	H257	+	/	/	/	+	+	+
	H258	+	+	+	+	-	-	-
	H259	+	+	+	+	-	-	+
	H260	+	+	+	-	+	-	-
	H221	+	+	-	-	-	+	+
	H213	+	+	-	-	-	+	-
Positive rate		58.33%	100.00%	50.00%	50.00%	0.00%	25.00%	8.33%
$10^{2.43}EID_{50}$ number = 12	L481	-	+	-	+	-	-	-
	L482	+	+	+	+	-	-	-
	L483	+	+	-	+	-	-	-
	L484	-	+	+	-	-	-	-
	L485	+	+	-	-	-	-	-
	L486	-	+	+	-	-	-	-
	L487	+	+	+	-	-	-	-
	L488	-	+	-	+	-	+	-
	L489	+	+	+	-	-	-	-
	L490	+	+	-	+	-	+	+
	L491	+	+	-	-	-	-	-
	L492	-	+	+	+	-	+	-
Positive rate		91.67%	100.00%	50.00%	50.00%	25.00%	16.67%	8.33%
$10^{1.43}EID_{50}$ number = 12	Z81	+	+	-	-	-	+	-
	Z82	+	+	+	+	-	-	-
	Z83	+	+	+	+	-	-	-
	Z84	+	+	-	-	+	-	+
	Z85	+	+	+	+	-	-	-
	Z86	-	+	-	-	-	-	-
	Z87	+	+	+	-	-	-	-
	Z88	+	+	-	-	+	-	-
	Z89	+	+	+	+	-	-	-
	Z90	+	+	-	+	-	-	-
	Z216	+	+	+	-	-	-	-
	Z217	+	+	-	+	+	+	-
Control	B486	-	-	-	-	-	-	-
	B487	-	-	-	-	-	-	-
	B488	-	-	-	-	-	-	-
	B489	-	-	-	-	-	-	-
	B490	-	-	-	-	-	-	-
	B71	-	-	-	-	-	-	-
	B72	-	-	-	-	-	-	-
	B73	-	-	-	-	-	-	-
	B74	-	-	-	-	-	-	-
	B75	-	-	-	-	-	-	-

during cell culture passage, with only the CEO homogenate inducing CPE in LMH cells, characterized by cell nuclear fusion and desquamation of cell layer (Figure 1). Subsequent infection of LMH cells with harvested progeny virus failed to elicit CPE, and blind serial passage indicated an inability of HBHG2023 to establish productive infection in vitro, with rapid reduction in viral titers after 3 passages (Table 5). These data reveal attenuated pathogenicity of HBHG2301 in cultured cells, with failure to sustain infection, which implying defects in the cell-derived viral progeny of efficient replication and induction of cytopathology (Qiao et al., 2020). Further systematic genomic and pathogenic analyses are needed to elucidate the underlying mechanisms.

Currently, only 1 serotype of ILTV has been reported. PCR-RFLP methods have been used to divide ILTV into different genotypes, but these remain difficult to distinguish from existing vaccine and virulent field strains (Choi et al., 2016). The glycoprotein gJ is unique to the envelope of ILTV and shares no homology with other herpes viral glycoproteins (Yang et al., 2020). Variations in gJ can provide ample information for distinguishing between different viral strains (Craig et al., 2017). Phylogenetic analysis of the gJ gene was undertaken in this study to elucidate the background of isolate HBHG2301 and its relationship between vaccine strain or local field strains (Figure 2). The full-length gJ sequence of HBHG2301 exhibited highest homology to

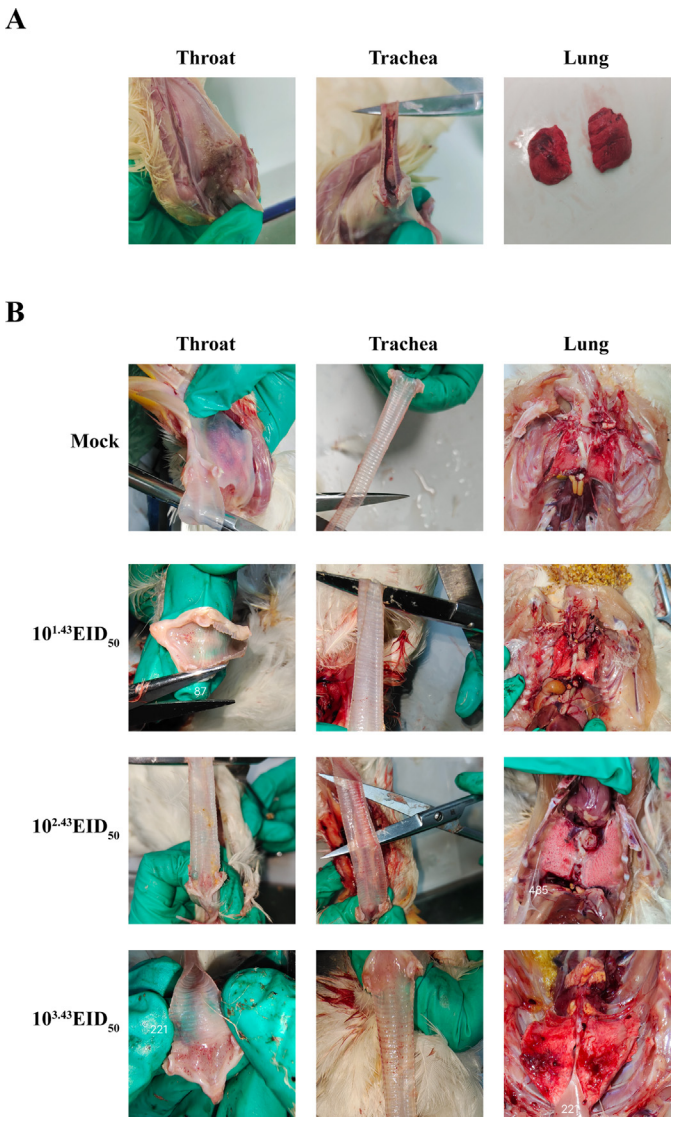

Figure 4. Necropsy examination and documentation of gross lesions of HBHG2301 infected chickens (A) 3 of 15 died in third day postinfection. (B) Fourteenth day postinfection.

the domestic 2012 isolate LJS09 and the uncommonly used vaccine strain Serva, with amino acid alignment revealing a unique T441P mutation not previously observed in available sequences or reports (Table 4; as of May 2023 in NCBI database). The widespread circulation of ILTV, coupled with frequent pressure mutations and recombination events with vaccine strains, presents challenges for the control of ILTV in the context of globalized poultry farming (Craig et al., 2017; Fakhri et al., 2020). Scholars worldwide have expressed concerns regarding the current use of live attenuated ILTV vaccines. Inappropriate vaccine application poses an even greater threat to poultry farms than wild-type viruses (Perez-Contreras et al., 2021; Santander-Parra et al., 2022). Given these circumstances, it is crucial to closely monitor T441P mutation described in this study, as it has the potential to drive the evolution of infectious bronchitis and contribute to pathogenic changes, which was needed to be further elucidated.

To date, detailed functional studies on ILTV gJ protein remain scarce. Glycoprotein gJ is a late protein encoded by the US5 gene, with 4 isoforms of varying molecular weights expressed via alternative splicing of US5 mRNA transcripts (Fuchs et al., 2005). gJ exhibits potent immunogenicity, inducing both humoral and cellular immunity in vivo (Veits et al., 2003). Deletion of gJ resulted in significantly higher intracellular vs. extracellular viral titers and larger plaques compared to the wildtype virus, suggesting an important role for gJ in virion egress from infected cells (Mundt et al., 2011). Besides, the extensive O- and N-glycosylation of gJ is also associated with virion maturation and egress (Fuchs and Mettenleiter, 2005). Additionally, US5, US6, and US7 comprise a unique late gene transcription cluster potentially involved in regulating late protein expression, although the mechanisms remain elusive. In the current study, sequencing confirmed the presence of a unique T441P mutation in the gJ protein of HBHG2301 isolate, in the central region of gJ (see Table 4). This mutation involves the substitution of a polar amino acid (threonine) with a nonpolar amino acid (proline), which has the potential to affect the structure of the gJ protein. Specifically, proline is known to enhance the stability of protein secondary structures, particularly in terms of thermal stability (Prajapati et al., 2007; Wrapp et al., 2020). Additionally, whether T441 undergoes phosphorylation during infection and its function remain unknown. Consequently, we cannot ascertain if the limited in vitro growth in LMH cells and pathogenicity in susceptible hosts observed for this isolate directly relate to the T441P mutation. However, mutations in surface immunodominant antigens of highly conserved herpesviruses warrant our greater attention and investigation regarding effects on viral immunogenicity. Further research into the origin and evolution of gJ may enrich our understanding of the mechanisms underlying ILTV's adaptive infection and propagation under various conditions.

Previous study has established an ocular infection model in 7-day-old chicks using 10^3 TCID50 ILTV, resulting in detectable viral loads among multiple tissues by 7 days postinfection (Tran et al., 2021). The highest viral loads were observed in conjunctival tissues, with relatively comparable levels in the kidneys, lungs, spleen, and trachea, etc. While ILTV replication is believed to occur predominantly in tracheal and conjunctival epithelial cells, not all ILTV strains could establish infection and cause pathology within pulmonary tissues (Zhao et al., 2013; Reddy et al., 2014). An interesting observation in this study was the higher pulmonary viral detection rate compared to the throat and trachea in the $10^{3.43}$ EID_{50} HBHG2301 infection group at 14 days postinfection (Table 6), despite limited lung pathogenesis upon necropsy (Figure 4). We proposed 2 potential explanations for this phenomenon. First, we hypothesized that the absence of characteristic clinical symptoms in most chickens from this group indicates that they have successfully overcome the acute ILTV infection, resulting in a gradual viral clearance from the upper respiratory tract. This persistence of the virus may be attributed to a delicate balance between limited pulmonary viral replication and host immunity (Rodriguez-Avila

et al., 2007; Wang et al., 2013). Alternatively, it was possible that HBHG2301 had undergone tropism shifts, gradually attenuated its virulence and expanded its tissue tropism. This could be a result of natural and vaccine selection pressures, enabling enhanced viral propagation and dissemination (Sabir et al., 2020). Further studies were required to determine if the HBHG2301 gJ T441P mutation was related to this hypothesis, as well as to investigate other potential recombination events or mutations (Gowthaman et al., 2020). Nonetheless, the pathogenic characteristics displayed by HBHG2301 warrant concern regarding ILTV evolution and variation.

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DISCLOSURES

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

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