Original Research

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Molecular characterization and identification of Infectious Laryngotracheitis Virus from clinical samples of poultry flocks in Indonesia

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ABSTRACT

Infectious Laryngotracheitis (ILT) in chickens is a significant issue that demands attention in Indonesia. This highly contagious respiratory disease caused by herpes requires urgent study and action. Despite the lack of confirmed ILT virus (ILTV) infection in layer hens in Indonesia, it is crucial to understand and address this potential threat. This study was dedicated to detecting and characterizing ILTV in layer hens from Bogor, Bekasi, Cianjur, and Tangerang districts for the first time in Indonesia, utilizing robust molecular techniques, including polymerase chain reaction (PCR) and sequencing. Three-layer farms in Bogor, Bekasi, and Cianjur districts, Indonesia, suffered a severe ILT outbreak, and one layer farm in Tangerang district, Indonesia, was declared slightly affected by ILT disease. Clinical and PCR assays were used to diagnose and report all layer farms. There have been reports of this illness in Indonesia, and more recently, outbreaks have been observed in the provinces of Bogor, Bekasi, and Cianjur. The present study used PCR and DNA sequencing methods to examine the ILTV. The work used organs and pooled tracheal swabs from clinically infected and dead chickens to target the envelope gG gene of ILTV. According to the analyses, 15 out of 48 suspicious field samples had isolated positive results. DNA sequencing results revealed that the amplified segment resembled the gene being studied. Information regarding the existence of ILT disease in laying chickens exhibiting respiratory symptoms during the outbreak is provided by this study.

Introduction

The highly contagious and severe Infectious Laryngotracheitis Virus (ILTV), caused by a group of herpes viruses, can affect chickens of all ages (Gowthaman *et al*., 2020). Poultry producers may suffer large financial losses as a result of ILT because of higher mortality, lower egg output, and the expenses of managing and controlling disease (Ghalyanchi *et al*., 2020). In extreme situations, the symptoms include coughing, gasping, bloody mucus discharge, and severe breathing difficulties that may lead to suffocation (Rojs *et al*., 2021). ILTV infection causes respiratory disease symptoms in various bird species, such as chickens, pheasants, partridges, and peafowl (Tsiouris *et al*., 2021). Two main types of ILT in hens have been identified in their natural environments (Ou and Giambrone, 2012). The severe acute variant is characterized by expectoration of blood-mixed mucus, sneezing, and severe respiratory discomfort (Kaur, 2021). Conversely, the milder type is typified by mild to severe conjunctivitis, sinusitis, and catarrhal tracheitis (Gowthaman *et al*., 2020). Pigeons, starlings, crows, sparrows, and ducks exhibit resistance to ILT (Mossad *et al*., 2022).

Researchers have been exploring the prevalence, incidence, pathogenicity, immunity, spread, and diagnostic methods of ILTV (Thilakarathne *et al*., 2020). There is an urgent need to quickly detect and protect the chicken population from these diseases due to the prevalence of transmitted diseases and the ensuing financial losses for poultry producers (Ghalyanchi *et al*., 2020). Indonesian poultry producers currently employ imported ILT vaccination to prevent ILT without taking local isolates or ILTV serotypes into account. This virus has been confirmed in various countries, namely Asia, Europe, South America, and North America. Partadiredja *et al*. (1982) documented the disease's initial appearance in Indonesia, and Indriani *et al*. (2002) created an enzyme-linked immunosorbent assay to identify ILT viral antibodies in commercial hens from several Indonesian regions. Additionally, ILTV infection has been documented in commercial broiler flocks based on clinical and pathological data (Indriani *et al*., 2004).

Clinical and pathological findings have since been used to document ILTV infection in commercial broiler flocks (Rojs *et al*., 2021). The presence of ILTV in Indonesian poultry flocks has yet to be confirmed using essential molecular methods such as PCR and sequencing (Chashmi *et al*., 2021). These methods are crucial for determining the disease's molecular epidemiology and tracing the virus's origin. Thus, the purpose of this study was to use nucleotide sequencing and PCR-based detection techniques to isolate, identify, and characterize ILT in Indonesian poultry flocks. This research aimed to provide insight into improved ILT control and vaccine development in Indonesia.

Materials and methods

Ethical approval

The commission approved the collection of swab and tracheal samples from chickens for Experimental Animal Research Ethics, National Research and Innovation Agency (Approval Number 166/KE.02/SK/08/2023).

Farms used

Four commercial layer chicken farms in the Bogor, Bekasi, Tangerang, and Cianjur districts were carefully inspected and observed for any indications of ILTV disease. The farms were specifically chosen based on information provided by Livestock Services, ensuring a comprehensive and targeted approach to the observation process. None of the chicken farms had ever had an ILTV vaccination, and every farm we visited had a history of clinical symptoms like ILT illness. All of the farms employed various

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combinations of immunizations against Newcastle Disease (ND), Infectious Coryza, Infectious Bursal Disease (IBD), and fowl pox. The affected birds ranged in age from 9 to 56 weeks. No information was available on the vaccination history of layer chickens with ILT.

Sample collection

Research and sampling were conducted from March 2023 to August 2023. From four farms, 48 tracheal and lung tissues and throat swabs were gathered. The samples were taken from birds showing signs of respiratory distress. A viral transport medium comprising 1000 µg/ml of Streptomycin (Meiji), 1000 IU/ml of Penicillin (Meiji), and 50 µg/ml of Gentamycin (SIGMA) was added to these samples. All of the samples were transported to the lab in an ice box. Upon arrival at the laboratory, they were promptly moved to a –80°C freezer until processing. Specific Pathogen-Free (SPF) embryonated chicken eggs (9–12 days old) were obtained from PT. Vaksindo Satwa Nusantara (PT. Vaksindo) to isolate and propagate the ILT viral samples. Every sample used five embryonated eggs.

Virus isolation

To create a 10% suspension that was clarified at 1,600 x g for 15 minutes, the trachea and larynx samples were frozen, thawed three times, and then pulverized in a sterile mortar and pestle. A 0.45 µm Millipore filter was then used to filter the supernatant. Then, 0.2 ml of the supernatant was added to each of the six chorioallantoic membranes (CAMs) of embryos that were 10–12 days old. To create a filtered suspension, the trachea and larynx samples were frozen, thawed, pulverized, and cleared. The trachea and larynx samples were frozen, thawed, ground, and clarified to obtain a filtered suspension of embryonated chicken eggs. The inoculated eggs were then incubated for six days at 37°C. In order to collect the virus, the eggs were stored at 4°C for 12 hours. Then, they were cleansed and opened in a sterile manner. The embryos were also chilled at 4°C for 24 hours. The pocks that developed on the CAMs resembled little pocks that were often encircled by a translucent edematous zone, a depressed gray core area of necrosis, and whitish or yellowish pocks with opaque margins. Over three processing and passages were performed on the CAMs free of pock blemishes. The sample was deemed negative for ILTV if, following the third passing, the CAMs did not exhibit pox lesions. The pox-lesioned CAMs were crushed and suspended in 10% Dulbecco's Modified Eagle Medium (DMEM) from GIBCO.

Ten minutes at 4°C were spent centrifuging the 10% CAM suspension at 1,000 x g. The Newcastle disease virus (NDV) was then detected in the supernatant by rapid hemagglutination (HA). 10% chicken red blood cells were combined with ILTV supernatant on a plate for this test. Any supernatant that did not agglutinate after 15 minutes was deemed negative for NDV and kept until it was needed at –80°C.

Polymerase chain reaction and sequencing

Following the cutting of tissue samples into tiny pieces, 5% homogenates were made in phosphate-buffered saline (pH 7.2). The DNeasy Blood and Tissue Kit (Qiagen) was used to extract DNA in compliance with the manufacturer's instructions. According to Ottiger (2010), the PCR was conducted using the primer pair ILTp32 U2-CTA CGT GCT GGG CTC TAAT CC and ILTp32 L2-AAA CTC TCG GGT GGC TAC TGC, which target the envelope glycoprotein-G (gG) gene (US4 gene) of GaHV-1. The reaction will use HotStar Taq Master Mix (Qiagen, Cat. No. 203443). A final extension will take place at 72°C for 10 minutes after denaturation at 95°C for 15 minutes, followed by 35 cycles at 95°C for 1 minute, 61°C for 1 minute, and 72°C for 1 minute. Two microliters of the PCR product will undergo electrophoresis in a 1.5% agarose gel after the PCR amplification. Any samples producing the expected 588 bp will be considered positive.

The products of PCR were exctracted using PCR Purification Kit (Qiagen, Cat. No. 28104). The PCR result was then sequenced on an Applied Biosystems 3130 DNA Sequencer using the BigDye Terminator v3.1 kit. Then, using reference sequences from GenBank, phylogenetic and molecular evolutionary analyses were carried -out using MEGA version 11 (Tamura *et al*., 2021). The Maximum Likelihood approach with Kimura-2P correction and 1,000 bootstrap replications was used to do the investigations and evolutionary relationships.

Results

Clinical signs and lesions

Four commercial poultry layer farms in Bogor, Bekasi, Tangerang, and Cianjur districts (Table 1) experienced symptoms similar to the ILTV. Apart from Farm III in Tangerang district, all three farms (Farm I, II, and IV) had similar disease histories, characterized by a sudden onset, marked dyspnea, and high mortality. Birds from 8 to 25 weeks old were affected, with those between 12 and 25 weeks of age showing slight susceptibility. Affected birds displayed recumbency, closed eyes, forward and upward head positioning during breathing, spasmodic coughing with blood-stained mucous expulsion, and nocturnal coughing, sneezing, and wheezing (Figure 1). Farms III and IV reported a drop in egg production by 2 to 15%, and Farm I observed poor shell quality eggs. Swollen faces, wattles, conjunctivitis, and frothy ocular discharge were also noted. The illness lasted for 2 to 3 weeks in a poultry house with a 5,000-bird capacity, with morbidity rates ranging from 20 to 30% and mortality rates from 12 to 17%.

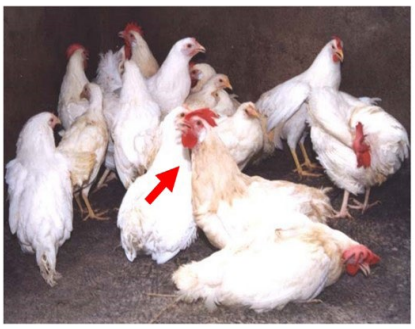

Fig. 1. The most frequent respiratory symptoms were coughing, gasping, and trouble breathing (Arrowhead).

Table 1. The population, age, vaccination history, illness, and mortality rates of birds from four farms that may have infectious laryngotracheitis.

Code of farm/Location	Population	Breed of chicken	Age (week)	Morbidity (%)	Mortality (%)	Vaccinations	Clinical Signs*
Farm I/Bogor	1,200	Hy-Line	8	10	8	ND, IBD, Fowl Pox	Cg, Sz, Gs, D, La, Lw, Nd, C, L
Farm II / Bekasi	5,800	Star-Cross	25	30	17	ND, IBD	D, La, Dp, En, Lw, C, Nd, Rep
Farm III/Tangerang	1,300	Hy-Line	12	0	0	ND, IBD, Fowl Pox	Ncs
Farm IV/Cianjur	5,000	Hy-Line	12	20	12	ND, IBD, Coryza	Cg, Sz, Gs, La, Lw, Rep, Dp, En

Note: Clinical Signs: C = Conjunctivitis; Cg = coughing; D = Dyspnea; Dp = Depression or stand with wing spread; En = Extended neck during an inpirating effort; Gs = Gasping; L = Lacrimation or eatery eyes; La = Lost appetite; Lw = Loss of body weight; Nd = Nassal discharge; Rep = Reduce egg production; Sf = Sweeling face; Sz = Sneeing

Pathological examination

The pharynx, larynx, and trachea were the only areas of the afflicted birds that had lesions. Particularly in birds from farms I and II, the trachea and laryngeal mucus had elevated blood flow and minor hemorrhages (Figure 2). The respiratory system was overloaded with mucus, and caseous tracheal plugs were observed, primarily in the top part of the trachea but sometimes along the entire length. Some birds developed mild tracheal irritation and a lot of mucus. The buccal mucous membrane had a yellow outer layer that was easily removed, exposing raw, inflammatory tissue underneath. Additionally, the eyelids exhibited numerous signs of inflammation associated with conjunctivitis. However, conjunctivitis and eyelid irritation were uncommon symptoms that were only seen in three cases on all farms.

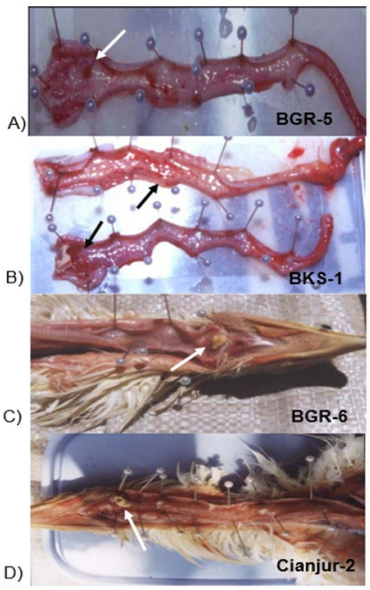

Fig. 2. A) and B) tracheas showing petechial hemorrhages collected from Farm I sample BGR-5 and Farm II sample BKS-1; C) and D) Caseous tracheal plug observed in the tracheas from Farm I sample BGR-6 and Farm IV sample CJR-2 (Cianjur-2).

Isolation

The experiment employed chicken eggs that had been embryonized using Specific Pathogen Free (SPF). Some eggs were inoculated with the Laryngo-Vac cover strain (Zoetis) as a positive control, and other eggs were injected with minimum necessary medium (MEM) as a negative control. The CAMs of the eggs were checked for pock lesions following a six-day incubation period. CAMs were gathered and transferred into additional 6- to 9-day-old SPF embryonated eggs, both with and without lesions. The CAMs showed the development of pock lesions during the second egg passage after being inoculated with the vaccination virus.

Conversely, there were no pock lesions on the eggs that were infected with MEM. For visual reference (Figures 3A and 3B). Forty-eight samples were collected from four commercial layer chicken farms and tested for isolation—fifteen out of 48 isolated samples tested positive. In cutaneous adverse drug reactions (CADRs), the skin lesions typically manifest as pustules raised with whitish or yellowish coloring. Indicative of tissue necrosis, these pustules have sunken gray center regions and opaque margins (Table 2 and Figure 4). The size of each pock varied from 1 to 3 mm, and large regions of pock coalescence were also seen.

Samples BGR-1, BGR-2, BGR-3, BGR-4, BGR-5, and BGR-6 were collected from Farm I Bogor District; samples BKS-1, BKS-2, BKS-3, and BKS-4 were collected from Farm II Bekasi District; and samples CJR-1, CJR-2, CJR-3, CJR-4, and CJR-5 were collected from Farm III Cianjur District. However, Farm IV (Tangerang District) samples showed negative results after three passages on CAM. The size of the CAM lesions ranged from tiny, dispersed foci to massive, 2 cm-diameter lesions. They were scattered around the CAM and were observed to grow in subsequent areas.

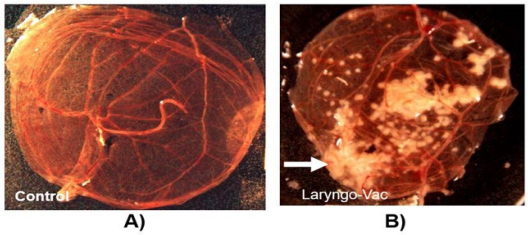

Fig. 3. There were both positive and negative ILT controls on CAMs. A) CAM infected with Laryngo-Vac Cover Strain (Zoetis) displayed pock lesions characteristic of ILT (arrowhead), acting as the positive control; B) CAM inoculated with MEM, the negative control, displayed no pock lesions.

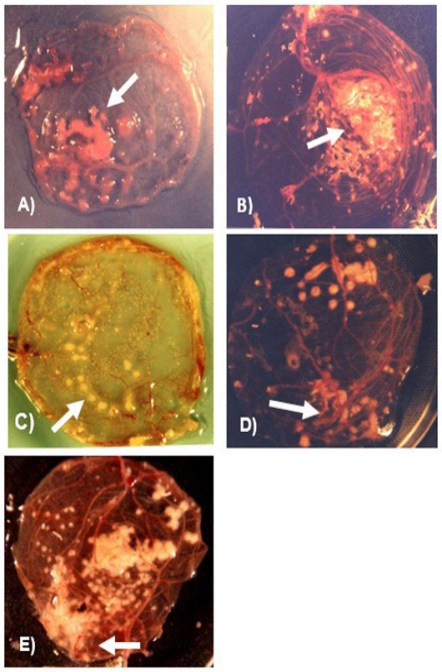

Fig. 4. Pock lesions on the third passage of the chorio allantoic membrane (Arrowhead). A) Isolate from Farm I #BGR-5; B) Isolate from Farm I # BGR-6; C) Isolate from Farm II # BKS-1; D) Isolate from Farm II # BKS-2; and E) Isolate from Farm IV# CJR-1. A, B, D, and E show large coalesced pock lesions distributed throughout the CAM and depressed gray central area of necrotic. C showing a few scattered pocks formed on the CAM.

Three passages in all were carried out for isolation. Samples were deemed negative if they did not develop pock lesions following the third passage. Pock lesions did not develop in the CAM in the second or third passages, and embryonic death before five days after injection was uncommon. The NDV was found in Farm I. Although no pock lesions were observed until the third passage, all of the samples from this farm were determined to be negative for ILTV. However, all embryonated eggs injected with samples from this farm were dead and showed signs of bleeding 48 to 72 hours after inoculation. The rapid plate hemagglutination test revealed that the CAM suspension from these eggs was positive for NDV. It is noteworthy that the injected embryonated eggs in none of the samples from the other farms displayed any indications of NDV lesions.

Table 2. Findings for ILTV isolation utilizing 10-day-old SPF embryonated eggs injected by the CAM method.

Code of Farm	Location	Number of samples tested	Results of isolation	
			Positive	Negative
Farm I	Bogor	13	6	7
Farm II	Bekasi	8	4	4
Farm III	Tangerang	12	0	12
Farm IV	Cianjur	15	5	10
Total		48	15	23

The AGID test

Results in Figure 5 showed a strong similarity line in all five ILTV isolates from Bogor District, Bekasi District, and Cianjur District. The positive control A high resemblance line was also seen in ILTV utilizing the vaccination strain (Laryngo-Vac, Cover Strain). The viral samples interacted with the particular ILT antisera after being diluted with buffer at a 1:2 ratio. The diluted test also showed identity lines toward the antigen-containing wells despite the dilution. These findings show that there is a correspondingly higher concentration of antibody to antigen.

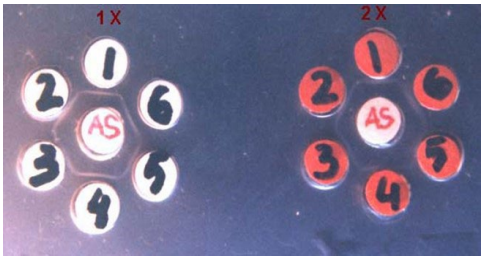

Fig. 5. The AGID test revealed an identification reaction against the antisera standard (anti-ILT strain NS175) for five ILTV isolates from the districts of Bogor, Bekasi, and Cianjur. The samples were undiluted (left side) and diluted at 1:2 (right side). Wells 1) isolate #BGR-5; 2) Isolate #BGR-6; 3) isolate #BKS-1; 4) isolate #BKS-2; 5) isolate #CJR-1; 6) control positive ILTV strain vaccine and AS (central well) antisera against ILTV strain NS-175.

PCR detection of ILTV and phylogenetic analyses

ILTV was detected in three out of four farms using PCR assay. The positive results were found in Farm I in Bogor District (BGR-1 to BGR-6), Farm II in Bekasi District (BKS-1 to BKS-4), and Farm IV in Cianjur District (CJR-1 to CJR-6). However, samples from Farm III in Tangerang District tested negative. An anticipated product size of 588 bp was obtained from the tissue samples' amplified partial envelope glycoprotein-G gene (US4 gene) (Figure 6). Upon sequencing, fourteen samples showed clear reads, which were used for further phylogenetic analysis. Nucleotide sequence analysis of envelope glycoprotein-G gene amplicons from fourteen field samples [ILT/BKS-1/WJ/Indonesia/2023 (GenBank: PV694685), ILT/BKS-2/WJ/Indonesia/2023 (GenBank: PV694686), ILT/BKS-3/WJ/Indonesia/2023 (GenBank: PV694687), ILT/BKS-4/WJ/Indonesia/2023 (GenBank: PV694688), ILT/BGR-5/WJ/Indonesia/2023 (GenBank: PV694689), ILT/BGR-4/WJ/Indonesia/2023 (GenBank: PV694690), ILT/BGR-1/WJ/Indonesia/2023 (GenBank: PV694691), ILT/BGR-2/WJ/Indonesia/2023 (GenBank: PV694692), ILT/BGR-3/WJ/Indonesia/2023 (GenBank: PV694693), ILT/CJR-1/WJ/Indonesia/2023 (GenBank: PV694694), ILT/CJR-2/WJ/Indonesia/2023 (GenBank: PV694695), ILT/CJR-3/WJ/ Indonesia/2023 (GenBank: PV694696), ILT/CJR-4/WJ/Indonesia/2023 (GenBank: PV694697), and ILT/CJR-5/WJ/Indonesia/2023 (GenBank: PV694698)] examined showed a 100% degree of homology with Brazilian GaHV-1 Laryngo-Vac (GenBank: FJ444830.1), GaHV-1 Isolate Nobilis ILT Brazil (GenBank: FJ444831.1), and GaHV-1 strain MS-LT BLEN China (GenBank: EU423894.1) reference strains.

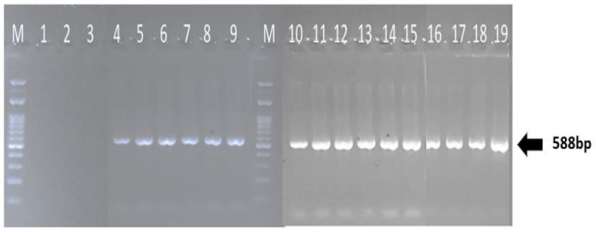

Fig. 6. PCR amplicons specific to the ILT virus that are visible through agarose gel electrophoresis. Lane M molecular weight marker; Lane 1 negative control; Lane 2-3 field samples showing negative result (TNG-1 and TNG-2); Lane 4-18 field samples showing the ILT virus-specific 588 base pairs product (BGR-1, BGR-2, BGR-3, BGR-4, BGR-5, BGR-6; BKS-1, BKS-2, BKS-3, BKS-4; and CJR-1, CJR-2, CJR-3, CJR-4, CJR-5); and Lane 19 Positive control.

The Indonesian ILTV percent identity matrix showed 99.10% homology with other reference strains and 99.16% homology with the Tunisian GaHv-1 isolate (GenBank: KY131964.1) (Table 3). The phylogenetic tree was built using 13 reference sequences from different nations and 14 partial envelope glycoprotein-G (gG) gene sequences. Laryngo-vac Brazil, Isolat Nobilis ILT Brazil, and Strain MS-LT-BLEN China were shown to be associated with Indonesian ILTV sequences (Figure 7).

Discussion

Only fourteen PCR-positive samples had their envelope gG gene nucleotide sequenced and aligned, and their identity percentage was compared to reference strains acquired from GenBank. The nucleotide sequence analysis of fourteen field samples shows that they are 100% homologous to the reference strain of GaHV-1 MS-LT BLEN China (GenBank: EU423894.1), GaHV-1 Laryngo-Vac Brazil (GenBank: FJ444830.1), and GaHV-1 Isolate Nobilis ILT Brazil (GenBank: FJ444831.1). The percent identity matrix calculated for Indonesian ILTV's shared 99,16 % homology with GaHv-1 isolate Tunisia (GenBank: KY131964.1), 99,10% homology with GaHv-1 Vac strain Intervet Italy (GenBank: 230798.1), GaHv-1 ILT CEO Vac Strain Intervet Italy (GenBank: HM230782.1), Strain ILT-77-

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
ILT/WJ/BKS-1/Indonesia/2023																											
ILT/WJ/BKS-2/Indonesia/2023	100.0																										
ILT/WJ/BKS-3/Indonesia/2023	100.0	100.0																									
ILT/WJ/BKS-4/Indonesia/2023	100.0	100.0	100.0																								
ILT/WJ/BGR-5/Indonesia/2023	100.0	100.0	100.0	100.0																							
ILT/WJ/BGR-4/Indonesia/2023	100.0	100.0	100.0	100.0	100.0																						
ILT/WJ/BGR-3/Indonesia/2023	100.0	100.0	100.0	100.0	100.0	100.0																					
ILT/WJ/BGR-2/Indonesia/2023	100.0	100.0	100.0	100.0	100.0	100.0	100.0																				
ILT/WJ/BGR-1/Indonesia/2023	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0																			
ILT/WJ/CJR-5/Indonesia/2023	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0																		
ILT/WJ/CJR-4/Indonesia/2023	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0																	
ILT/WJ/CJR-3/Indonesia/2023	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0																
ILT/WJ/CJR-2/Indonesia/2023	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0															
ILT/WJ/CJR-1/Indonesia/2023	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0														
FJ444830.1_GaHV-1_Laryngo-Vac_Brazil	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0													
HM230782.1_GaHV-1_ILT_CEO_vac_strain_Intervet_Italy	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10												
FJ444831.1_GaHV-1_isolate_Nobilis-ILT_Brazil	99.94	99.94	99.94	99.94	99.94	99.94	99.94	99.94	99.94	99.94	99.94	99.94	99.94	99.94	99.94	99.10											
KC248152.1_GaHV-1_isolate_Jiangsu_China	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.10	99.08	99.13										
KC248155.1_GaHV-1_isolate_Shandong_China	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.10	99.98									
KX344453.1_GaHV-1_strain_ILT.77.IR_Iran	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.10	99.15	99.15								
EU281342.1_GaHV-1_isolate_LVV15BR_gC_Brazil	99.13	99.13	99.13	99.13	99.13	99.13	99.13	99.13	99.13	99.13	99.13	99.13	99.13	99.13	99.13	99.08	99.13	99.23	99.23	99.08							
KY131964.1_GaHV-1_isolate_Tunisia	99.19	99.19	99.19	99.19	99.19	99.19	99.19	99.19	99.19	99.19	99.19	99.19	99.19	99.19	99.19	99.06	99.15	99.08	99.08	99.04	99.08						
MK089453.1_GaHV-1_isolate_ILT_Namibia	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.10	99.98	99.98	99.15	99.23	99.10					
MZ323228.1_GaHV-1_strain_B59-11_gE_UK	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.02	99.10	99.06	99.06	99.13	99.02	99.17	99.06				
LC592201.1_GaHV-1_Ma2_gG_Myanmar	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.02	99.10	99.15	99.15	100.0	99.08	99.04	99.15	99.13			
HM230798.1_GaHv-1_vac_strain_Intervet_Italy	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.08	99.06	99.10	99.15	99.15	99.13	99.08	99.00	99.15	99.15	99.13			
EU423894.1_GaHV-1_strain_MS-LT-BLEN_China	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.10	99.94	99.10	99.08	99.08	99.13	99.19	99.08	99.08	99.08	99.08	

Table 3. Percentage of nucleotide divergence of 27 ILT isolates calculated by MEGA 11 program software.

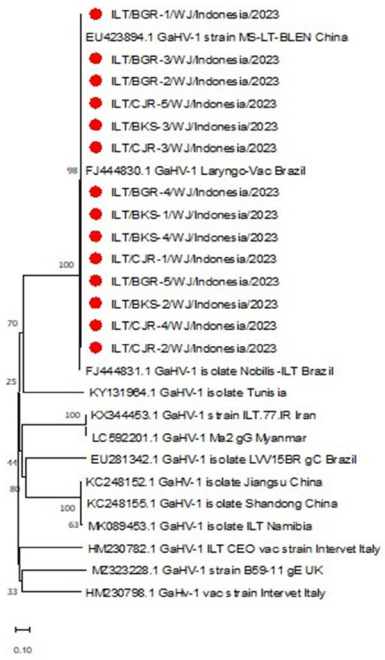

Fig. 7. A phylogenetic tree was created based on the partial sequences of the ILT virus envelope glycoprotein-G gene (US4 gene). Phylogenetic analysis was done using the MEGA 11 program. Multiple alignments of the envelope glycoprotein-G gene's (US4 gene) amino acid sequences were used to build the tree, and 1000 bootstrap replicates were used for neighbor-joining analysis. Red circles indicate the locations of our study samples.

IR Iran (GenBank: KX344453.1), GaHv-1 Ma2 gG Myanmar (GenBank: LC592201.1), GaHV-1 Isolate Shandong China (Gen Bank: KC248155.1), GaHV-1 strain B59-11 gE United Kingdom (GenBank: MZ323228.1), and GaHV-1 Isolate ILT Nambia (GenBank: MK089453.1). The envelope glycoprotein-G (gG) gene's 14 sequences were used to create the phylogenetic tree, including 13 reference sequences from different nations. Strain MS-LT-BLEN China, Isolat Nobilis ILT Brazil, and Laryngo-vac Brazil were shown to be associated with Indonesian ILTV sequences (Figure 7). According to our nucleotide and amino acid sequence analysis, ILTV vaccine strains may be the source of outbreaks in commercial layer chicken flocks in West Java, Indonesia. Nevertheless, unvaccinated flocks provided the samples. These results are consistent with those of (Hussein and Abdullah, 2022; Nourhan *et al*., 2019; El-Saied *et al*., 2021; Bayoumi *et al*., 2020), who found that the vaccinal strain was linked to the ILTV in outbreaks. Given that viruses from the CEO and TCO-ILT vaccines regain their virulence through bird-to-bird back passages, this suggested that they could trigger severe epidemics in Indonesian flocks that are already at risk. ILTV infection can be avoided by vaccination. Nevertheless, latently infected carrier chickens can be produced by ILT vaccination viruses. The virus can spread to flocks that have not received vaccinations thanks to these latent carriers (Hussein and Abdullah, 2022). Oldoni and García (2007) found that the vaccine strain closely matched most commercial chicken isolates of ILTV. These results are consistent with previous observations. These outbreaks have demonstrated that the wild vaccination strain is currently being replaced by the ILT circulating virus (Chang *et al*., 1997; Bayoumi *et al*., 2020). These results highlight how crucial it is to employ recombinant DNA-based vaccinations and put biosecurity measures in place to stop the spread of ILTV.

The existence of ILTV in Indonesia, namely among backyard hens, is anticipated to be examined by additional epidemiological research. The reported clinical illness in Canadian field cases was comparable to the observed clinical symptoms (Sary *et al*., 2017). The next step after detecting ILTV was to isolate it on Embryonated Chicken Eggs (ECE) through the Chorioallantoic Membrane (CAM) route. The viral isolation test is still considered the gold standard for diagnosing ILTV (Yu *et al*., 2020). Following the initial passage, the infected eggs developed yellowish-white pocks and widespread edema when the viral suspension was introduced into the ECE via the CAM channel. The membranes looked somewhat thicker and hazy compared to non-inoculated CAM. In contrast to our findings, which showed that pocks quickly developed on the CAM after the first passage, pock lesions were seen after two passages (Ibrahim *et al*., 2021), three passages (Magouz, 2015), and four passages (Islam *et al*., 2010). This may be explained by the high viral concentration and the virus's potent adaptation to ECE.

Conclusion

The diagnosis of ILTV in layer chickens from farms that had respiratory illness epidemics in Indonesia's Bogor, Bekasi, and Cianjur Districts has been validated by recent studies. Conducting epidemiological surveillance and figuring out the disease's incidence, prevalence, and economic impact will require more thorough research. Additionally, it's critical to determine the viruses' origins, distinguish between vaccine and field strains, and gather important data regarding the disease's genesis and modes of transmission. The evolutionary analysis of the outbreak's samples indicates that ILTV was introduced by carrier birds and quickly spread to other flocks, most likely as a result of the stressed-out non-vaccinated layers.

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Conflict of interest

The authors have no conflict of interest to declare.

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