2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 588-596.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj59
PII: S232245682500059-15

Clinical and Genetic Characterization of Infectious Laryngotracheitis Virus in Layer Chickens in Basrah, Iraq

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ORIGINAL ARTICLE
Received: June 24, 2025
Revised: July 26, 2025
Accepted: August 28, 2025
Published: September 30, 2025

ABSTRACT

Infectious Laryngotracheitis (ILT) has been widely prevalent in numerous countries in recent years. The present study aimed to detect ILT in chickens exhibiting clinical suspicion, utilizing molecular analytical techniques in Basrah, Iraq. Clinical signs, histological changes, and polymerase chain reaction (PCR) were used to diagnose thirty-five samples collected from clinically suspected chickens, including trachea and lungs for histopathology and PCR testing for the *p32* gene. The present study demonstrated that the three isolates of ILTV, including ILT-BASRAH 1, ILT-BASRAH 2, and ILT-BASRAH 3, caused the typical signs and pathological changes of ILT in layers chickens. Phylogenetic analyses indicated that ILT-BASRAH 1 exhibited a distant relationship with other current local isolates. Conversely, the isolates ILT-BASRAH 2 and ILT-BASRAH 3 exhibited a close relationship with each other. The initial results indicated that ILT-BASRAH 1 exhibited high nucleotide similarity to MK895003.1, MK895000.1, and MK894999.1 from Australia, which are representative of recombinant strains derived from live attenuated vaccine strains, as well as PQ232589.1 from Georgia. Furthermore, ILT-BASRAH 2 and ILT-BASRAH 3 were identified as vaccine-related isolates. These isolates demonstrated a high level of genetic similarity and a close phylogenetic relationship to the *Gallid alphaherpesvirus* 1 isolate TJ2019 (GenBank accession no. PP062931.1), originating from Chinese source flocks. The current study determined that ILT-BASRAH isolates 1, 2, and 3 exhibited genetic similarity, suggesting that they likely originated from the same or closely related sources. Their difference from vaccine strains, including the minor variation in ILT-BASRAH 1, might indicate regional viral adaptation.

Keywords: *Gallid alphaherpesvirus*, Histological change, Layer chicken, Phylogenetic

INTRODUCTION

Chickens are primarily affected by many respiratory diseases. Infectious Laryngotracheitis (ILT) was first identified in the United States in 1925. It continues to cause substantial economic damage to the worldwide poultry sector (Fuchs et al., 2007). The ILT is caused by *Gallid herpesvirus 1*, which belongs to the Herpesviridae family (Ou and Giambrone, 2012). Clinical signs of ILT include nasal discharge, conjunctivitis, coughing, gasping, reduced egg production, and bloody mucus discharge. The severity of ILT varies; in certain cases, it may lead to severe respiratory complications that could potentially result in suffocation (Bagust et al., 2000). Diseases caused by ILT result in significant economic losses. These losses occur as they have a direct impact on growth, mortality, and egg production (Oldoni et al., 2008). Reduced egg production is also a primary indicator in laying hens infected with infectious laryngotracheitis virus (ILTV), as the disease impacts the overall health of the chickens and results in significant stress (Tadese et al., 2007). ILTV is mainly transmitted horizontally through direct contact with infected chickens or by inhaling secretions and droplets. Additionally, contaminated tools, food, and water supplies help spread the infection within poultry flocks. However, no vertical or transovarial transmission through eggs has been documented (Pajić et al., 2022). Following the infection, the virus begins to replicate in the trachea and conjunctiva. Clinical signs of illness in hens generally manifest after five days (Dufour-Zavala, 2008). Infected chickens may begin to transmit the virus within two days of infection. Additionally, sick hens could act as a source of ILTV (Chukiatsiri and Pohuang, 2018). Despite vaccination of chicken flocks, ILT disease remains a threat, particularly in areas with a high poultry industry presence (Yan et al., 2016). Therefore, a strategy of careful and regular clinical monitoring and ongoing immunological assessment of chicken flocks should be implemented as part of early diagnosis and control (Mo and Mo, 2025). Primary diagnosis of ILT depended on clinical signs, macroscopic changes, and then laboratory testing, as well as histopathological and molecular identification. Histopathological examination can identify common lesions such as syncytial cells and intranuclear inclusion bodies,

To cite this paper: Mohammed SS, Khaleefah IA, Ali RAA, Najem HA, Almayahi WM, and Lateif BM (2025). Clinical and Genetic Characterization of Infectious Laryngotracheitis Virus in Layer Chickens in Basrah, Iraq. *World Vet. J.*, 15(3): 588-596. DOI: https://dx.doi.org/10.54203/scil.2025.wvj59

and it is frequently employed for diagnostic purposes. Molecular testing is regarded as the fastest, most dependable, and precise method for confirming the diagnosis (Coppo et al., 2013). Recently, many studies have highlighted the significance of molecular screening technology in monitoring poultry flocks for disease control and diagnosis (Gatica et al., 2023; Priya et al., 2023). Furthermore, different poultry diseases, particularly respiratory infections such as Avian Influenza (AI), Infectious Bronchitis (IB), and Newcastle Disease (ND), are challenging to diagnose through clinical assessment due to the similar signs exhibited by AI, IB, and ND. Consequently, differentiating between clinical and molecular diagnoses proved to be challenging (Kamal et al., 2024). Therefore, the most effective method for diagnosis and monitoring is to use molecular techniques, which should be employed to identify the specific type of ILTV and its origin, as well as to accurately characterize the genotypic virus through the sequencing of particular gene segments (Abebe et al., 2024).

Therefore, the present study aimed to detect ILTV in commercial layer farms in Basrah, Iraq, through the examination of clinical, pathological, histological, and phylogenetic data based on the *p32* gene.

MATERIALS AND METHODS

Ethical approval

The current study was conducted with the permission of the Ethics Committee at the College of Veterinary Medicine, University of Basrah, Iraq (Ref. No. 36/2023).

Clinical signs, gross lesions, and sample collection

The present study lasted one year, from October 2023 to October 2024. Seven poultry farms under observation housed flocks of white Lohmann layers. The farms had a population ranging from 60,000 to 90,000 laying hens, each 20 weeks of age and with an average weight of 1,405 grams. The laying hens were at the end of their incubation and rearing stages. Suspected chickens exhibiting tentative clinical signs consistent with ILT were observed during the study period and underwent a meticulous clinical examination to document all signs. Subsequently, the chickens were humanely euthanized in accordance with ethical guidelines and then subjected to a thorough necropsy procedure at the Department of Poultry Diseases and Veterinary Pathology, College of Veterinary Medicine, University of Basrah, Iraq (Najem et al., 2024). Eight tissue samples from the trachea and lung were taken from chickens that exhibited typical signs of ILT.

Each sample was divided into two sections. The first section was kept in a container with 5 mL of glycerol phosphate buffer 50% solution and stored at -20°C for polymerase chain reaction (PCR). The second section was fixed in a formalin 10% solution for histopathological analysis. These fragments were then preserved in a neutral buffered formalin 10% solution, dehydrated using a progressive series of alcohol solutions, cleared using a series of xylene solutions, and infiltrated with different grades of melted paraffin in an oven set at 56°C (Yavuz et al, 2018). The tissues were further preserved in paraffin using a rotary microtome. The sections were subsequently stained using the hematoxylin and eosin staining method (H and E), as described by Lateif et al. (2024).

DNA extraction and PCR amplification

Following the manufacturer's guidelines, viral DNA was extracted from layer hens suspected of having ILTV using a commercial kit (Wizard® Genomic DNA Purification Kit, Promega, USA). The primers ILTU2 5-CTA CGTG CTGGG TGGCT ACTGC-3 and ILT2 5-AAACTCT CGGG TGGCT ACTGC-3 were utilized to amplify a segment of the *p32* gene associated with ILTV (Alaraji et al. 2019), resulting in a 588 bp product. The total volume of the reaction was established at 50 µL, with the PCR mix (InvitrogenTM, USA) containing 5 µL of DNA to initiate the amplification. The reaction was conducted using a multigene thermal cycler (Labnet, USA). Denaturation occurred at 95°C for five minutes, followed by 35 cycles of one minute at the same temperature. The extension phase lasted for ten minutes at 72°C. Following electrophoresis on a 1.5% agarose gel, the PCR results were visualized under UV light.

Sequencing

By using the maximum likelihood method with the Tamura-Nei model (Tamura and Nei, 1993), the evolutionary history can be inferred. The tree with the highest log probability (-1080.35) is the one that was displayed. The Tamura-Nei model generated a matrix of pairwise distances, which were subsequently employed to automatically construct the initial tree(s) for the heuristic search. The Neighbour-Joining method was utilized, and the topology with the optimal log likelihood value was selected. The present study examined fourteen nucleotide sequences. The codons were located in the first, second, third, and noncoding regions. The entire dataset comprised a total of 543 sites. The MEGA11 version 11 development team in the United States carried out the evolutionary analyses (Tamura et al., 2021).

RESULTS

Clinical signs, gross lesions, and histopathology findings

During the study, one poultry house was suspected of being infected with ILT. This infected poultry farm housed a total of 60,000 laying hens. Some laying hens exhibited severe clinical signs, including bloodied beaks, coughing, gasping, bulging eyes accompanied by wet rales, extended heads, and mouth-breathing. However, a decrease in egg production and conjunctivitis were commonly observed. Additionally, sudden death due to asphyxiation resulting from complete obstruction of the trachea and larynx was observed (Figures 1 and 2).

The tracheal mucosa, which showed noticeable congestion with mucoid exudate, was among the overall gross lesions. Additionally, hemorrhage on the tracheal mucosal surface was observed in the deceased chickens (Figures 3 and 4). The histopathological analysis exhibited necrotic epithelial cells mixed with blood and tracheal mucus. This material can solidify, resulting in the formation of a caseous plug in the trachea, which may potentially lead to asphyxia. Microscopic examination of the eight samples taken from chickens with respiratory distress revealed lesions in four of them. These included epithelial erosion, sloughing, and infiltration of different inflammatory cells, such as lymphocytes, along with the presence of apoptotic and necrotic cells in the subepithelial layer. In addition, there was vascular congestion and the creation of a pseudomembrane (Figures 5 and 6). The lesions in the lung samples were characterized by a thickening of the alveolar wall and the presence of inflammatory cells. Additionally, swelling of capillaries in the parabronchus and congestion of veins were observed (Figures 7 and 8).

Figure 1. Trachea of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. Tracheal hemorrhage is obvious.

Figure 2. Trachea of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. Tracheitis is obvious.

Figure 3. Trachea of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. Blood-tinged mucous is obvious.

Figure 4. Trachea of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. A clot in the trachea is obvious.

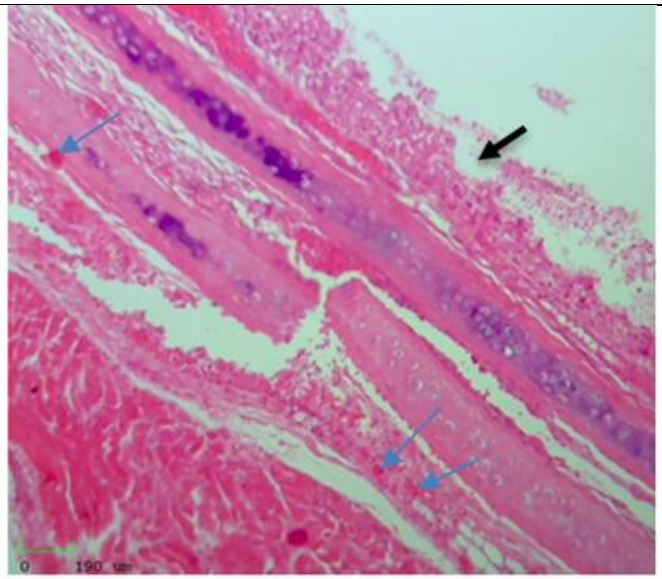

Figure 5. Trachea of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. Hemorrhagic tracheitis, mucosal damage (black arrow) with congestion in blood vessels (blue arrows, H&E x10).

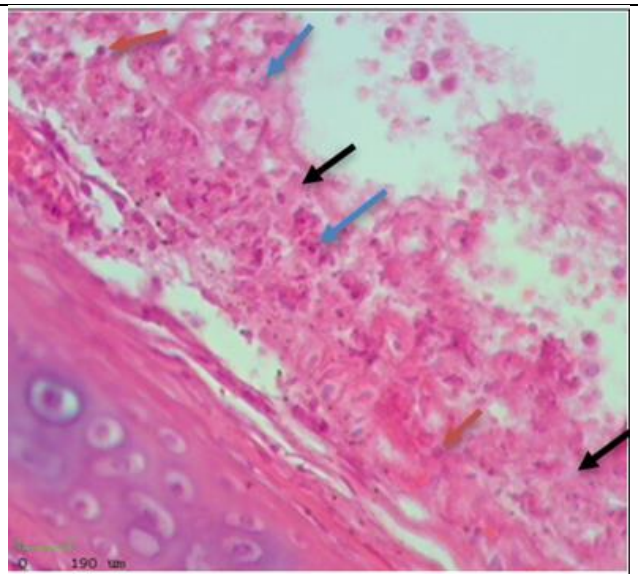

Figure 6. Trachea of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. Trachea mucosal layer with inflammatory cells (blue arrows), severe epithelial necrosis (black arrows), and intranuclear inclusion bodies (red arrows, H&E x40).

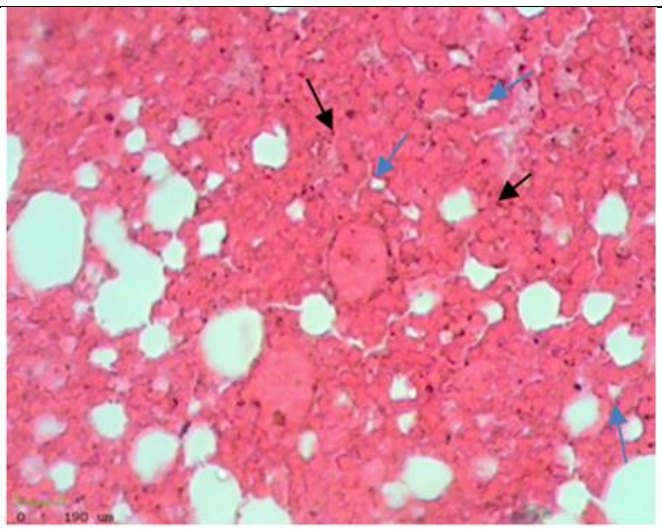

Figure 7. Lung of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. Lung thickening in the alveolar wall (blue arrows) with inflammatory cells in interstitial tissue (black arrows, H&E x40).

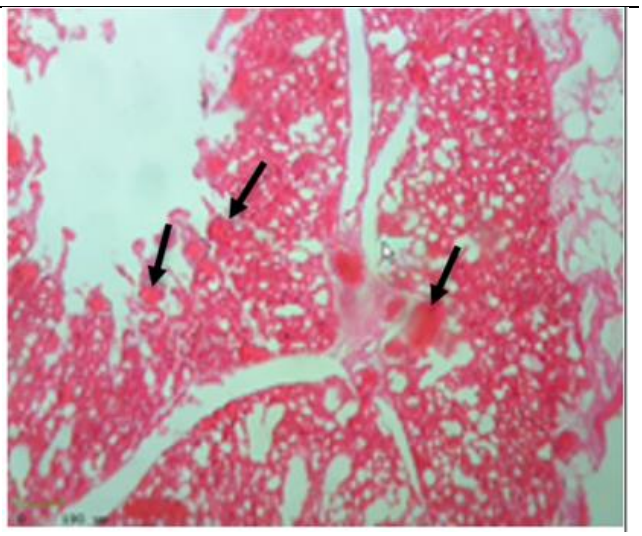

Figure 8. Lung of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. Lung engorgement of capillaries in para bronchus and venous congestion (black arrows, H&E x10).

PCR amplification

The PCR was performed to detect ILTV, which targeted the *p32 gene*, with a 588 bp product. Suspected samples were analyzed using a 1.5% agarose gel, stained with ethidium bromide, and subjected to UV light exposure. The favorable outcomes were labeled as 1, 2, and 3 (Figure 9). The bands signified successful amplification of the target gene. The positive control yielded precise results at the corresponding band level, thus corroborating the reaction's efficacy.

Sequence and bioinformatics analysis of the *p32* gene

Sequences in the current investigation were lined up and compared to those of reference strains, as shown in the phylogenetic tree isolates using the MEGA X program software (Figure 10). Creating a phylogenetic tree by analyzing the targeted gene sequence of the ILTV in three local isolates from Basrah, Iraq, and comparing them to strains listed in the GenBank database worldwide. The three local strains sent to the gene bank and assigned these names PP860566.1 (ILT-BASRAH 1), PP860567.1 (ILT-BASRAH 2), and PP860568.1 (ILT-BASRAH 3) had different genetic

relationships with the other strains. The ILT-BASRAH 1 was categorized within the same group as vaccine strains due to its substantial nucleotide similarity with specific global isolates reported in recent years, namely MK895003.1, MK895000.1, and MK894999.1. There was no genetic divergence among these isolates, indicating their close genetic relationship. This discovery suggested that the isolate may be closely related to vaccination strains or the result of a strain that has been spreading globally in the region recently. On the other hand, isolates ILT-BASRAH 2 and ILT-BASRAH 3 established a new evolutionary branch, with genetic distances of 0.034 and 0.010, respectively, from the other recorded strains in GenBank.

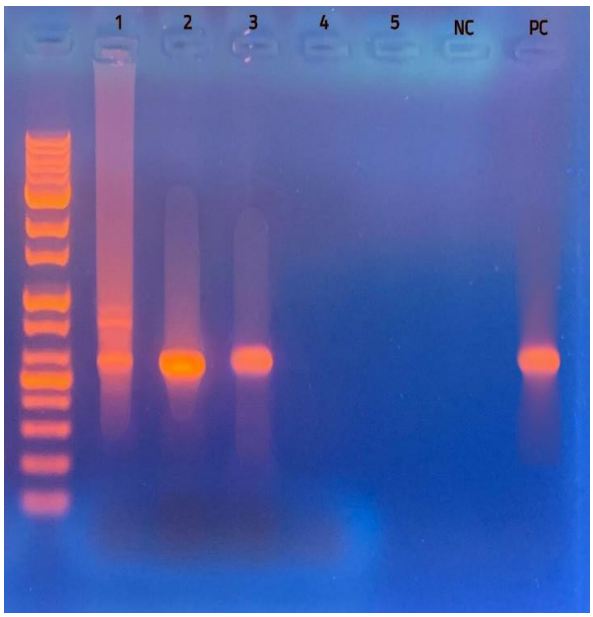

Figure 9. PCR results of the *p32* gene of Infectious Laryngotracheitis Virus. (1,2, and 3 field strains from layer chickens) were positive at the 588 base pair fragments. M=100bp DNA ladder; NC: Negative control; PC: Positive control

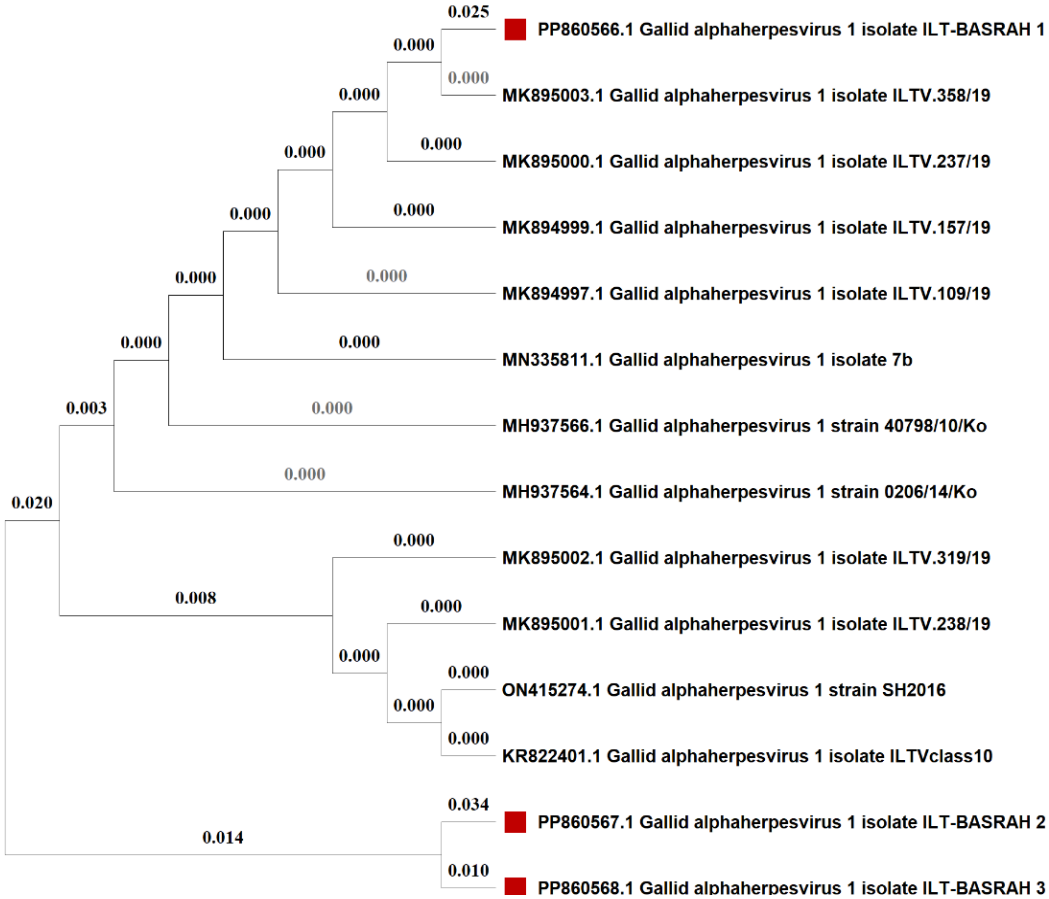

Figure 10. The phylogenetic tree of the infectious laryngotracheitis virus was isolated from a layer chicken's trachea and lung in Basrah, Iraq. Their accession numbers are used to express them in international nucleotide databases (Isolates using the MEGA X program software)

DISCUSSION

Avian ILT was identified as a specific viral disease affecting chickens within the global poultry industry (Marga et al., 2024). In Iraq, particularly in the southern region, such as Basrah Province, there is no available information regarding ILT.

The results of clinical monitoring demonstrated that one poultry house out of seven was diagnosed with ILTV. A similar result was mentioned by Gowthaman et al. (2020) and Kamal et al. (2024), who noted that the rate of infection depends on several factors, including the severity of the virus, the route of transmission among chickens, and the phase of the disease, such as acute or sub-acute infections. Another study on broiler chickens reported an infection rate of about 2% (Ali et al., 2023). The clinical observations in the present study included coughing, gasping, watery eyes with moist rales, blood in the beak, dyspnea accompanied by expectoration of blood-stained mucus, and conjunctivitis. These clinical observations were consistent with findings of García et al. (2013) and Rojs et al. (2021). Traditional descriptions of acute ILTV infection often mention rales, tracheitis, and gasping, which are linked to the involvement of the deeper layers of the upper respiratory tract and can cause tracheal obstruction.

The mortality rate in the present study was recorded as 1% which was in line with the findings of Roberts et al. (2011), who documented the virulence strain of the virus, causing death, resulting from mechanical asphyxiation caused by complete airway obstruction. The present results, which indicated that ILTV targets the respiratory system, agreed with those of Timurkaan et al. (2003) and Kaboudi et al. (2016), who reported that the virus causes pathological changes, including inflammatory changes, in the respiratory tract of infected chickens with ILTV. Similar lesions were observed by Kirkpatrick et al. (2006), who found hemorrhagic lesions in the trachea, tracheal blood clots, and congestion of the infra-orbital sinuses. These pathological changes result from rapid viral replication within the respiratory epithelium, leading to cell destruction, acute inflammatory reaction, and infiltration of inflammatory cells.

The microscopic alterations in the trachea demonstrated epithelial erosion and sloughing, along with the infiltration of many inflammatory cells, such as lymphocytes and apoptotic cells, in the subepithelial layer. Vascular congestion and the production of a pseudomembrane, which was caused by fibrin and dead cells, were observed. This finding aligned with the results reported by Carnaccini et al. (2022) in the trachea, larynx, sinuses, and conjunctiva. The appearance of lesions in the lungs exhibited that the ILT has progressed to a more advanced stage, as the virus spreads into the deeper respiratory tissues.

The severity of lesions differed based on the tissue affected and the virus's tropism. Certain ILTV strains demonstrated a high propensity to invade the trachea (tracheotropic strains), whereas their capacity to infect the lungs was less prominent. Genetic variations among strains may result in different virulence and preferred sites of replication within the organism (Perez-Contreras et al., 2021; Mo and Mo, 2025).

The molecular analysis of suspicious samples indicated positive solid PCR bands. The present study provided molecular information on the *Gallid alphaherpesvirus* strains that are currently disseminating within the poultry industry and contributed to a deeper understanding of the transmission dynamics of ILTV in Basrah, Iraq. All data regarding local strains indicated that the *p32* gene-targeted PCR test was a reliable and accurate method for determining the presence of *Gallid alphaherpesvirus* in poultry 1. These findings are consistent with those of Callison et al. (2007) and Oldoni and García (2007), who demonstrated the potential use of virus-specific genes for rapid and reliable diagnosis. The results of the phylogenetic analysis indicated that the ILT-BASRAH 1 did not exhibit close relatedness to any other existing local isolates (Figure 10).

ILT-BASRAH 2 and ILT-BASRAH 3 demonstrated a close relationship with each other. The local isolates in the present study exhibited varying genetic structures and are classified into two distinct clusters. The initial results indicated that the recombinant strain, ILT-BASRAH 1, showed high nucleotide similarity to MK895003.1, MK895000.1, and MK894999.1 from Australia, which represent recombinant strains derived from live attenuated vaccine strains, and PQ232589.1 from Georgia. It is noteworthy that recombinant strains of ILT have been documented in laying hens currently being studied for the first time in Iraq. Live attenuated vaccines have proven to be highly effective in lowering mortality rates, alleviating clinical signs, and preventing ILTV outbreaks (Fulton et al., 2000). In addition, a wide range of recombinant vaccines have been developed or are currently under investigation. These vaccines include ILTV strains with targeted deletions in virulence-associated open reading frames (Schneiders et al., 2018), as well as recombinant vectors such as fowlpox virus and turkey herpesvirus engineered to express specific ILTV glycoprotein genes (Johnson et al., 2010). In the present study, the second cluster of ILTV, ILT-BASRAH 2 and ILT-BASRAH 3, was identified as vaccine-related isolates. These isolates exhibited high genetic similarity and a close phylogenetic relationship to the *Gallid alphaherpesvirus* 1 isolate TJ2019 (GenBank accession no. PP062931.1), which was from China. *Gallid alphaherpesvirus* 1 was grouped within the chicken embryo origin and vaccine strain lineage. Furthermore, ILT-BASRAH 2 and ILT-BASRAH 3 indicated a phylogenetic association with the Georgia isolate MF417811.1. According

to Zhang et al. (2025), high-throughput sequencing conducted in China between 2015 and 2019 revealed that six ILTV isolates (SD2015, GD2017, SYB2018, HB201812, HB201806, and TJ2019) clustered together with chicken embryo origin vaccine strains. There have been significant concerns regarding the virulence and proportional risk of altering the virulence of classical live attenuated vaccines, despite their potential to provide high protection. Live attenuated vaccine strains, particularly CEO strains, can cause slight clinical signs in chickens, depending on the vaccine strain and the age of the chickens. Additionally, in poultry breeding facilities with a lot of chickens, live attenuated vaccinations can become virulent again (Guy et al., 1991).

Whole-genome sequencing technology can assist in mapping genomic feature differences and may explain these complex phylogenetic differences. The current findings are consistent with previous studies by Ojkic et al. (2006) and Moreno et al. (2010), showing that genetic sequence analysis can distinguish between vaccine-derived and field strains and reveal their evolutionary backgrounds. In the present study, phylogenetic analysis indicated that specific local ILTV isolates exhibited genetic variations distinct from those available in GenBank. These differences highlighted the potential for local viral evolution within Basrah, Iraq.

CONCLUSION

The present study concluded that the ILT-BASRAH 1, 2, and 3 isolates were genetically similar and likely originated from the same or closely related sources. These isolates are geographically separated from certain global vaccine strains, indicating minimal recent recombination or genetic variation from vaccines in these local isolates. The ILT-BASRAH 1 exhibited a slightly higher divergence from some of the reference isolates, suggesting possible unique mutations or evolution in local flocks. Comprehensive epidemiological surveillance across additional Iraqi provinces and border areas is essential to map the distribution and dynamics of ILTV strains for further studies.

DECLARATIONS

Ethical considerations

The authors have checked the ethical considerations, including issues such as plagiarism, consent for publication, misconduct, data fabrication and/or falsification, as well as double publication and/or submission redundancy.

Funding

The authors have borne all expenses. The initial research and genomic isolation procedures were carried out at the laboratories of the University of Basrah, Iraq.

Availability of data and materials

The data collected during this study are available from the corresponding author upon reasonable request.

Acknowledgments

The authors thank the staff of the University of Basrah's College of Veterinary Medicine for providing the necessary assistance and resources for the study project.

Authors' contributions

Sara Salim Mohammed reviewed and edited the manuscript. Isam Azeez Khaleefah contributed to the molecular detection. Rajaa Abd Alzahra Ali wrote the original draft. Harith Abdulla Najem contributed to the research design and field diagnosis of the disease. Waleed Majeed Almayahi performed the genetic analysis of the results. Budoor Muhammad Lateif conducted the histopathological study. All the authors read and approved the final version of the manuscript.

Competing interests

The authors declared no conflict of interest.

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