**Special Issue:**

Veterinary Medicine between Sustainable Development and Public Health to Confront Global Changes

# Studies of Respiratory Viral Infection in Chickens with Special Reference to Infectious Laryngotracheitis

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**Abstract** | Respiratory viral infections have a considerable detrimental impact on animal welfare and significant financial influences on the poultry industry. Avian influenza virus (AIV), Newcastle disease (ND), and infectious bronchitis (IB) are the most economically significant illnesses impacting the poultry sector worldwide, including Egypt. Also, infectious laryngotracheitis (ILT) may cause massive financial losses. The current study was designed to investigate the prevalence and co-infection dynamics of some viral etiological agents of respiratory problems in chicken flocks in Egypt from 2020 to 2021. Samples were collected from 69 broiler flocks across four governorates (Giza, Fayoum, Menya, and Menoufia) and investigated using RT-PCR to detect viral pathogens. Results showed that most flocks had been co-infected with more than one viral agent and there were 24 positives (35%) for IBV, 39 positives (57%) for H9N2, 18 positives (26%) for NDV, and 9 positives (13%) for ILTV. The results revealed a high prevalence of mixed infections, with 52% of flocks co-infected by two or more viruses. The most common co-infections involved IBV with H9N2 (9%) and IBV with ILTV (9%). Genetic analysis of the ILTV isolates indicated a 97–100% nucleotide identity with previously reported Egyptian field strains, confirming the circulation of endemic ILTV strains. Phylogenetic analysis also showed close clustering with TCO vaccine strains, highlighting the genetic stability of the virus. These findings highlight the need for improved biosecurity, vaccination strategies, and continuous molecular surveillance to control respiratory diseases in Egyptian poultry.

**Keywords**: Respiratory viral infection, Broilers, ILT, Mixed infection

**Received** | June 26, 2024; **Accepted** | August 01, 2024; **Published** | November 13, 2024
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**Citation** | Orbano AE, El-Dimerdash M, Abaza M, Zanaty A, Rady M, Nemr M, El-Feil WK (2024). Studies of respiratory viral infection in chickens with special reference to infectious laryngotracheitis. Adv. Anim. Vet. Sci. 12(s1): 395-403.
**DOI** | https://dx.doi.org/10.17582/journal.aavs/2024/12.s1.395.403
**ISSN (Online)** | 2307-8316; **ISSN (Print)** | 2309-3331



## INTRODUCTION

The worldwide poultry sector, a crucial component of food security, has persistent challenges from viral diseases, especially those impacting the respiratory system of the birds. Viral respiratory diseases incriminated in a great economic loss to the global poultry industry due to reduced productivity, increased mortality rates, and the cost of control measurements (Hafez and Attia, 2020). The dominance of the intensive poultry production system and global trade in addition to migratory birds facilitate the spread of several viral respiratory pathogens in rearing flocks all over the world, frequently subsequent co-infections with more than one pathogen that exacerbate the severity of

infections (Gržinić *et al*., 2023).

Avian Influenza Virus (AIV), Newcastle Disease Virus (NDV), Infectious Bronchitis Virus (IBV), and Infectious Laryngotracheitis Virus (ILTV) are some of the most significant viral infections affecting the world's poultry industry. These worldwide spread viruses cause respiratory problems which can range from mild to severe, and finally lead to significant losses in production in all poultry sectors (Ramzy, 2016). Commonly occurring co-infections involving two or more of these viruses' complicate diagnosis, treatment, prevention and control efforts. The low pathogenic H9N2 subtype of the Avian Influenza Virus is the predominant virus detected in chicken populations globally, including in Egypt. Although H9N2 is less perilous than H5 and H7, it can still result in considerable production losses and elevated mortality when co-infected with other pathogens. Newcastle Disease Virus, which is a highly contagious avian Orthoavulavirus, continues to cause significant morbidity and mortality despite extensive vaccination measures (Bhat *et al*., 2022). The Infectious Bronchitis Virus (IBV), a coronavirus that predominantly infect the respiratory and reproductive systems of chickens, is significant in the poultry industry's broiler and layer sectors (Najimudeen *et al*., 2020).

Infectious Laryngotracheitis (ILT) is a chicken respiratory tract infection caused by Gallid herpesvirus type 1 (GaHV-1) that affects broilers, pullets, and adults and reduces weight gain and egg production. Gasping, bloody mucus expectoration and high mortality characterise severe epizootic ILT. Mucoid tracheitis, sinusitis, unthriftiness, and low mortality characterise mild, sometimes enzootic infections. ILT is distributed worldwide, but it may only be present in certain localities within a country or geographic region or in specific (multiple-age) production sites, whether they are industrial or backyard flocks (Gowthaman *et al*., 2020). ILT is first reported in Egypt in 1983 (Tantawi *et al*., 1983) and repeated virus isolation and serological survey studies have proved the widespread existence of clinical and subclinical forms of the disease among layer and broiler flocks. After that, many outbreaks of ILT were reported in Egypt. ILTV is an enveloped virus with an icosahedral nucleocapsid and a 150 kbps linear ds-DNA genome. It has a Unique Long (UL) and Unique Short (US) region (Perez *et al*., 2020).

Herpes viruses produce pathogenically significant proteins, including envelope, tegument, infected cell protein 4 (ICP4), capsid, glycoproteins, TK, transcriptional regulator, and non-structural proteins. The ILTV genome has 80 ORFs, 65 in the UL, 9 in the US, and 6 in the IR. The ILTV ORF that encodes ICP4 is 4386 nucleotides long and has 59% GC, starting from the first of four ATG codons. It weighs 175 kDa. ICP4 and alpha herpes virus IPC4 share two highly homologous domains. ICP4 contains four translational start codons based on Kozak 1986's sequence at nucleotide (nt) locations 2514, 2811, 2835, and 2847. In early and late replication, ICP4 initiates transcription (MacLachlan and Dubovi, 2017).

In Egypt, where the poultry industry is a cornerstone of the agricultural economy, respiratory viral infections present a continuous challenge. The co-circulation of H9N2, NDV, IBV, and ILTV in Egyptian poultry flocks has been documented, often resulting in severe outbreaks with increased mortality and production losses. The ability of these viruses to complex co-infections that can also mask clinical signs and hinder effective disease diagnosis (Hegazy *et al*., 2019; Yehia *et al*. 2021, 2023). The present study was aims to assess the prevalence of key respiratory viruses in Egyptian commercial broiler farms from 2020 to 2021, with a particular focus on Infectious Laryngotracheitis (ILT) and its genetic characterizations.

## MATERIALS AND METHODS

### Sample collection and preparation

The study was carried out between 2020 and 2021 on commercial broiler chicken farms in Egypt's four governorates: Giza, Fayoum, Menya, and Menoufia. A total of 69 broiler farms were selected based on reports of respiratory distress and mortality, with birds exhibit clinical symptoms such as coughing, sneezing, gasping, and ocular and/or nasal discharge. Flocks with varying ages and production types were included in this study to cover a broad representation of the local poultry industry.

Ten birds displaying characteristic symptoms of respiratory illness were chosen for sampling from each flock. Tracheal, choanal cleft, laryngeal, and lung tissues were collected from each bird during necropsy. Samples were immediately placed in 50% buffered phosphate buffer saline, stored at -80°C, and transported to the laboratory for further diagnostic testing. Another copy of the samples was fixed in 10% neutral buffered formalin For histopathological examination. Postmortem examination was performed to assess any gross pathological changes, including congestion, hemorrhage, and the presence of blood clots in the trachea and infraorbital sinuses. The tissue samples were thawed before processing and homogenized using a sterilized pestle and mortar in sterile phosphate-buffered saline (PBS) with gentamycin (50 µg/mL), penicillin (2000 units/mL), streptomycin (2 mg/mL), and mycostatin (1000 units/mL) to produce a 10% W/V suspension. The homogenates were clarified by centrifugation at 3000 rpm for 30 minutes.

### Nucleic acid extraction and real-time PCR (RT-PCR)

Total viral DNA and RNA were extracted from the processed

tissue samples using the Viral Gene-spin™ Viral DNA/RNA Extraction Kit (iNtRON Biotechnology) according to the manufacturer's instructions. A combination of DNA and RNA extraction was employed to accommodate both RNA viruses (such as AI, IBV, and NDV) and DNA viruses (such as ILTV). The quality and concentration of the extracted nucleic acids were assessed using a Nano Drop spectrophotometer. To detect the presence of viral pathogens,

Real-time PCR (RT-PCR) assays were performed using specific primers and probes designed for AIV, NDV, IBV, and ILTV. The primers and probes were selected based on previously published sequences (Spackman *et al*., 2002; Wise *et al*., 2004; Callison *et al*., 2006; Shabat *et al*., 2010; Ou *et al*., 2012). As shown in Table 1. The rRT-PCR was performed as a one-step format using AgPath-ID™ One-Step RT-PCR Kit (Applied BiosystemsTM). The reaction mixture was prepared containing: 7.5µl of 2X RT-PCR Buffer, 6 pmol of each forward and reverse primers and 2 pmol of each TaqMan® probe, 0.6 µl of 25X RT-PCR Enzyme Mix, 2µl volume of template and RNAse-free water to reach 15 µl. The amplification program consisted of a first reverse-transcription step at 45°C for 10 min, followed by 10 min at 95°C (hot start) and 45 PCR cycles of denaturation at 95°C for 10s, annealing and extension at 60°C for 45s. The reaction was carried out using Swift™ Spectrum 48 Real-Time PCR Detection System. The fluorescence emitted by FAM, JOE, Cy3 and Texas Red dyes was measured simultaneously and independently at the end of the annealing step.

## Nucleotide sequencing and phylogenetic analyses

Isolates from positive ILTV samples were subjected to genetic characterization using primers ICP4-F:5'-GGGTCTTGTTCTGCAGGATTCT-3' and ICP4-R:5'-TGCTACCTGGAGAATGTCCCGATG – 3' to amplify a 620-bp fragment (Molini *et al*., 2019). The PCR was performed in an applied biosystem thermal cycler (ProFlex™ PCR System) using Emerald Amp GT PCR Master Mix (Takara, Kusatsu, Japan). The negative control was PCR master mix, primers, and PCR-grade water. Specific PCR products were separated by electrophoresis in a 1.5% agarose gel stained with ethidium bromide using a Biometra® Compact electrophoresis system (Analytik, Jena) and visualized using a Biometra® gel documentation system (UV star 312 nm - Biometra Laboratories, Milan, Italy), and the QIAquick Gel Extraction Kit was used to purify the positive PCR fragments (Qiagen, Hilden, Germany).

The nucleotide sequence was obtained using an ABI 3500XL Genetic Analyzer (Life Technologies, California, USA), and the identical primers listed above, as well as the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA). The sequences of five isolates were compared to those of other ILTV strains representing various groups and vaccine strains being used in Egypt

**Table 1:** Specific primers and probes used for AIV, NDV, IBV, and ILTV identification.

| Virus | Target gene | Oligo | Sequence (5-3) | Reference |
|---|---|---|---|---|
| AIV | Matrix protein (M) | F | AGATGAGTCTTCTAACCGAGGTCG | (Erica *et al*., 2002) |
| | | R | TGCAAAAACATCTTCAAGTCTCTG | |
| | | Probe | TCAGGCCCCCTCAAAGCCGA | |
| AIV-H9 | HA | F | GGAAGAATTATTTATTGGTCGGTAC | (Ben Shabat *et al*., 2010) |
| | | R | GCCACCTTTTTCAGTCTGACATT | |
| | | Probe | ACCAGGCCAGACATTGCGAGTAAGATCC | |
| H5 | HA | F | ACG TAT GAC TAT CCA CAA TAC TCA G | (Erica Spackman *et al*., 2002) |
| | | R | AGA CCA GCT ACC ATG ATT GC | |
| | | Probe | TCA ACA GTG GCG AGT TCC CTA GCA | |
| NDV | F | F | AGTGATTGTCTCGGACCTTC | (Wise *et al*., 2004) |
| | | R | CCTGAGGAGAGGCATTTGCTA | |
| | | Probe | TTCTCTAGCAGTGGGACAGCCTGC | |
| IBV | S1 | F | GCTTTTGAGCCTAGCGTT | (Callison *et al*., 2006) |
| | | R | GCCATGTTGTCACTGTCTATTG | |
| | | Probe | CACCACCAGAACCTGTCACCTC | |
| ILTV | ICP4 | F | CCCCACCCAGTAGAGGAC | (Ou *et al*., 2012) |
| | | R | CGAGATACACGGAAGCTGATTT | |
| | | Probe | CAGTCTTTGGTCGATGACCCGC | |

which were obtained from the NCBI. The CLUSTAL-W tool and the MegAlign module of DNASTAR software were used to perform the alignment (Lasergene version 7.2; DNASTAR, Madison, WI, USA). MEGA version 6 was used to create the phylogenetic tree (Tamura *et al*., 2013), using the maximum likelihood approach with moderate strength and 1000 bootstrap repetitions (Kumar *et al*., 2016). DNAstar software (DNAStar, Madison, WI) was used to compute the pairwise nucleotide percept identity (Tamura *et al*., 2013).

### Histopathological examination

For histopathological analysis, fixed tracheal tissue samples in 10% neutral buffered formalin from ILTV- H9 positive birds were dehydrated through ascending grades of ethanol and embedded in paraffin. After embedding in paraffin, tissues were cut into sections of 4 µm thickness and were stained with hematoxylin and eosin for routine histopathological examination. Images were captured at magnification powers of 40x, 200x, 400x, and 600x using an Olympus BX43 microscope equipped with an Olympus DP27 digital camera with CellSens dimensions software (Olympus, Tokyo, Japan).

## RESULTS AND DISCUSSION

### Prevalence of respiratory viruses by molecular diagnosis

As shown in Figure 1 the epidemiological situation of respiratory viruses of each governorate is complex as real-time PCR analysis identified the presence of multiple respiratory viruses, with a high incidence of co-infections. 75% of Samples collected from Menoufia showed infection by one or more respiratory pathogens, Fayoum showed 84%, Giza had 88%, and Menya displayed 50%. In Menoufia, 42% of the sampled flocks were found to have mixed infections, with 25% of the flocks showing co-infections of IBV and ILTV, while 17% were co-infected with NDV and H9N2. In Fayoum, 51% of flocks had mixed infections, with 33% showing H9N2 dominance, and 17% co-infected with both NDV and H9N2. Giza had a high incidence of mixed infections at 66%, with 44% involving IBV and H9N2. In Menya, the situation was more varied, with 50% of the flocks testing negative for any viral infection, while 25% were co-infected with NDV and H9N2. These findings highlight the significant presence of mixed viral infections, complicating the diagnosis and management of respiratory diseases in Egyptian poultry.

Among all samples from four governorates, the total incidence of individual viral infections indicated that 57% of the examined flocks were positive for H9N2. That confirm the H9N2 is the most widespread virus in these areas. Thirty-five percent of the flocks tested positive for IBV, twenty-six percent for NDV, and thirteen percent for ILTV. Remarkably, 52% of examined flocks are co-infected with two or more viruses, with the predominant combination (9% of the flocks) being co-infection of IBV with H9N2 or IBV with ILTV. Additionally, 4% co-infected by H9N2 with ILTV, 3% co infected by H9N2 with IBV, and 6% H9N2 with NDV. Notably, all 9 ILTV-positive flocks (13%) were co-infected with either IBV or H9N2, highlighting the complexity of viral interactions in these cases. These findings emphasize the prevalence of mixed infections in Egyptian poultry, complicating both diagnosis and diseases control.

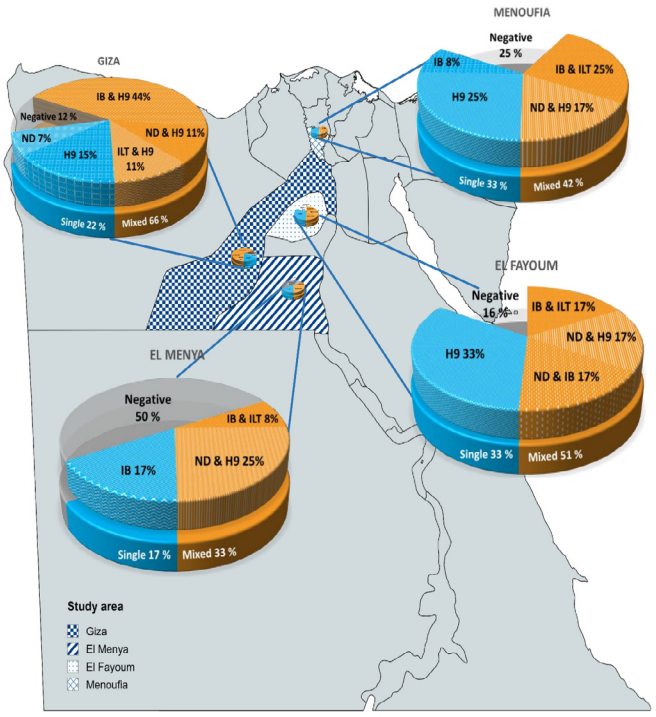



**Figure 1:** Epidemiological situation of tested respiratory files in four Egyptian governorates Giza, El-Menya, EL Fayoum, and Menoufia.

### Histopathological examination

Histopathological examination of tracheal tissues from ILTV-H9 positive flocks consistently revealed characteristic lesions associated with ILT. As shown in Figure 2. The severe fibrino-hemorrhagic tracheitis, observed in all testes samples which characterized by sever sloughing of the tracheal epithelium along with mononuclear cell infiltration. Ulceration and necrosis of the mucous glands were frequently report leading to further epithelial degeneration. Moreover, intranuclear eosinophilic inclusions, a characteristic of herpesvirus infections, were observed in 60% of the samples. The occurrence of multinucleated giant cells was recorded in 50% of the samples, associated with fibrinous exudates and hemorrhage in the tracheal lumen. These histopathological changes explain the respiratory distress and elevated mortality observed in ILTV-infected flocks.

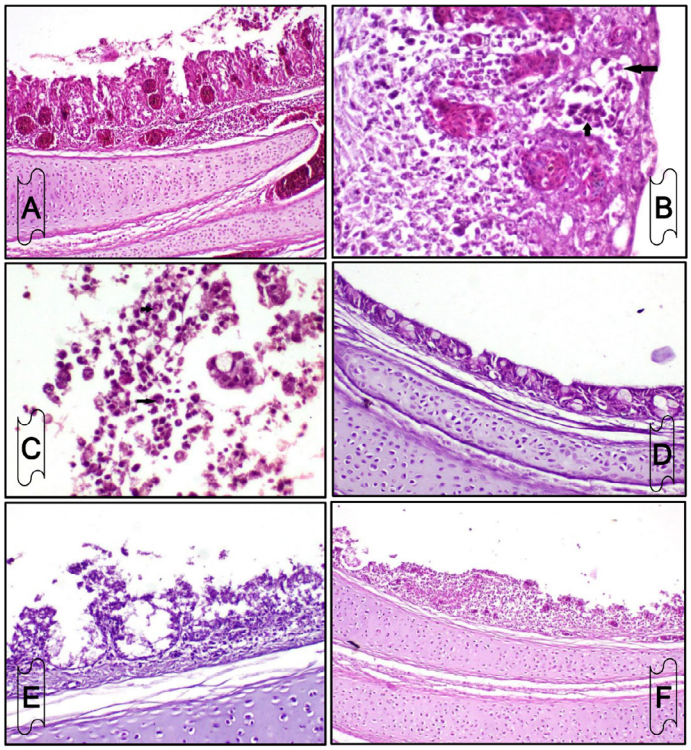



**Figure 2:** Histopathological examination of tracheal tissues from ILTV-positive flocks. A: Trachea of chicken D21 showing congestion of blood vessels and mononuclear inflammatory cells infiltration in the submucosa (H and E stain X 200). B: Trachea of chicken showing Intranuclear eosinophilic inclusions in epithelial cells (arrows) with blood vessels congestion and mononuclear inflammatory cells infiltration in the submucosa (H and E stain X 600). C: Trachea of chicken showing multinucleated syncytial cells and intranuclear eosinophilic inclusions in epithelial cells surrounded by a halo (arrows) (H and E stain X 600). D: Trachea showing an intact mucociliary mucosa with no signs of inflammation in the submucosa (H and E stain X200). E: Trachea of chicken showing sloughed epithelial lining, degeneration of mucous glands and mononuclear cells infiltration in the submucosa (H and E stain X400). F: Trachea of chicken in group KFG showing sloughed epithelial lining, and mononuclear cells infiltration in the submucosa (H and E stain X200).

## Genetic characterization of ILTV

To study the genetic diversity of ILTV strains circulating in Egyptian broiler flocks, five ILTV-positive samples underwent partial sequencing of the ICP4 gene. The strains' sequences were deposited in the GenBank database with accession numbers PQ356237, PQ356238, PQ356239, PQ356240, and PQ356241. The sequenced 620-bp fragment revealed 97–100% nucleotide identity with previously reported Egyptian field strains, indicating the continued circulation of endemic ILTV strains within the region. Comparisons with commercial vaccine strains, revealed 95–100% similarity, suggesting a close genetic relationship between field and vaccine strains.

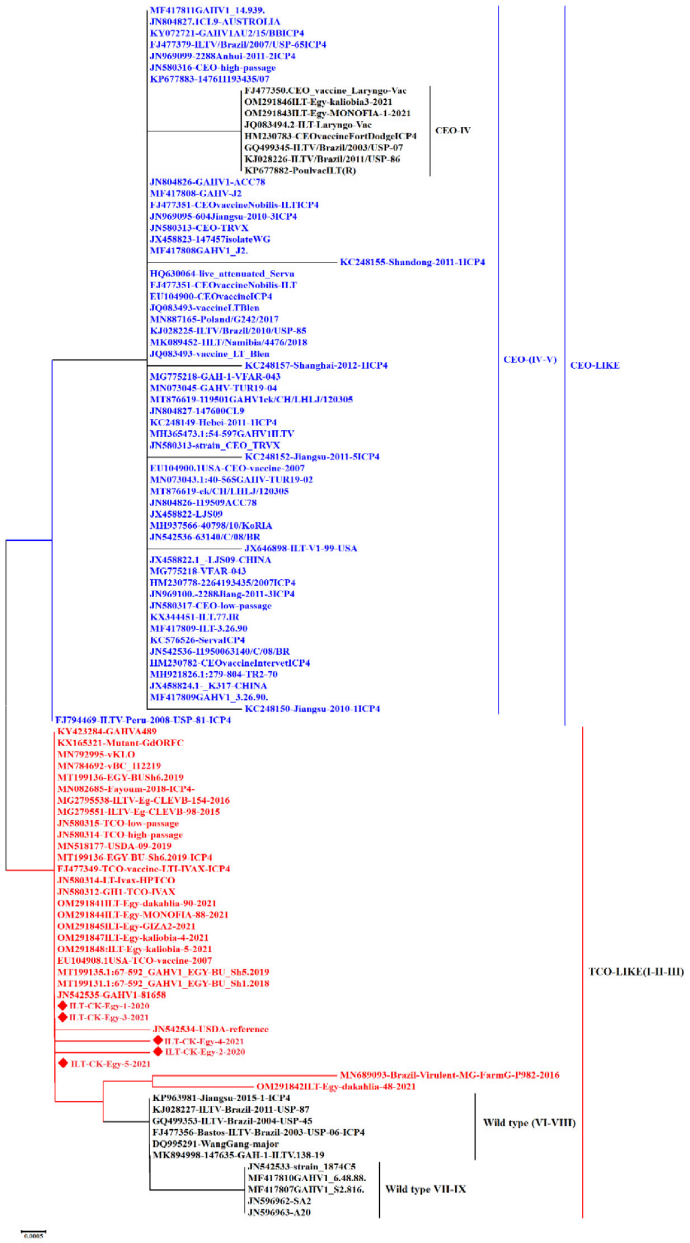
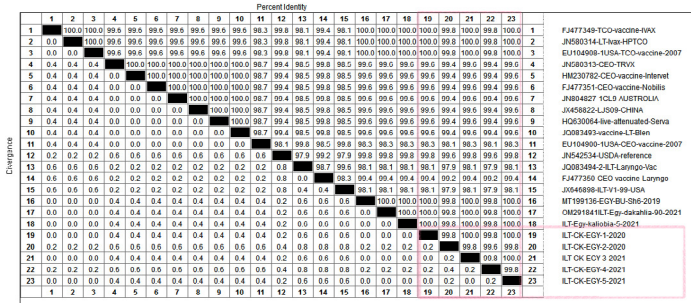



**Figure 3:** Phylogenetic analysis of strain under study compared with different field and vaccine strains of ILTV. The phylogram was generated by alignment of 620-bp fragment of ICP4 using the maximum-likelihood method in the MEGA 6.0 program. The name, origin, date of identification. The Egyptian strains identified in this study are indicated by a red asterisk. The CEO and TCO clades are indicated at the right side by red and blue brackets, respectively. Bootstrap probabilities are shown at the branch nodes. The scale bar at the bottom indicates the error rate.

Phylogenetic analysis confirmed that the Egyptian ILTV isolates clustered closely with other local field strains from previous outbreaks, (Figure 3). Although the genetic distance between the field isolates and vaccine strains was relatively small, the phylogenetic tree showed clear differentiation between the Chicken Embryo Origin and Tissue Culture Origin vaccine strains and the Egyptian field isolates. Further analysis of the ICP4 gene did not reveal

significant mutational changes in the Egyptian ILTV field isolates compared to vaccine strains as shown in Tables 2 and 3. The nucleotide positions, such as 536, 301, and 435, showed no substantial variation between the field and vaccine strains, indicating a high level of conservation in this region. Additionally, the amino acid sequence alignment at positions 91–95 (AAQDV motif) showed no variation among the Egyptian strains, suggesting that the ICP4 gene remains relatively stable across both field and vaccine strains.

**Table 2:** Amino acid identities of ICP4 gene sequenced compared to other selected Egyptian strains and reference vaccine strains.




The epidemiological study of respiratory viral infections in Egyptian broiler farms located in four governorates between 2020 and 2021 reveals the prevalence of mixed infections, offering significant challenges to efficient diseases control. Our findings show a high incidence of H9N2, IBV, NDV, and ILTV, with combined infections affecting more than half of the flocks. These findings are consistent with broader reports from the Middle East and North Africa (MENA) region (Hegazy *et al*., 2019; Hassan *et al*., 2021).

The current investigation found that H9N2 was the most common pathogen, detected in 57% of examined flocks. This is consistent with recent findings on the prevalence of H9N2 in the Egyptian poultry production industry, where it has been associated to large economic losses due to its potential to co-infect with other respiratory viruses and bacteria, resulting in increased pathogenicity (Ramzy, 2016). The ability of H9N2 to potentiate co-infections with IBV, NDV, or bacterial agents significantly strengthens clinical symptoms, leading to increased morbidity and mortality rates (Yehia *et al*., 2021). Co-infection between H9N2 and other respiratory pathogens has been a recurring problem in Egyptian poultry farms, where mixed infections with IBV and NDV have been commonly observed (Samy and Naguib, 2018).

Recent studies emphasize that H9N2, despite being classified as a low pathogenic avian influenza, exacerbates the effects of other respiratory viruses like IBV, increasing the severity of clinical manifestations in poultry (Pan *et al*., 2012; Gržinić *et al*., 2023). In our study, co-infections of H9N2 with IBV were found in 9% of flocks, supporting previous observations that these two pathogens often act synergistically in respiratory disease complexes (Najimudeen *et al*., 2020). This underlines the necessity for multifaceted control strategies combining vaccination and improved biosecurity measures.

Even though ILT is not a main disease for broiler chickens and is typically not vaccinated against, its frequency varied throughout the four governorates, with an overall detection rate of 13%. Specifically, 25% of flocks in Menoufia had ILT and IBV co-infections, whereas 17% had both ILT and H9N2. In Fayoum, 17% of flocks were infected with both ILT and H9N2, highlighting the complexity of viral interactions in mixed infections. The high rate of co-infections in these places shows that ILT frequently circulates in tandem with other viral diseases. These findings match with prior studies by Mossad *et al*. (2022), who also identified ILT epidemics in Egyptian broiler during the same period, 17.5% of broiler flocks tested positive for ILT, supporting the widespread occurrence of ILT in Egyptian broiler poultry farms.

**Table 3:** The nucleotide position was calculated from the start codon of the ICP4 gene.

| | Isolates | Amino acid position | | | | | Nucleotide position | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| S. | SDC position | $^{91}$AAQDV$^{95}$ | 101 | 180 | 199 | 227 | $^{272}$CGGCCCAAGACG$^{283}$ | 261* | 301 | 435* | 536 |
| 1 | U104908.1USA-TCOvaccine-2007 | ----- | L | R | V | N | ----- | C | C | G | G |
| 2 | EU1049001-USA-CEO-vaccine-2007 | deleted | - | - | M | - | deleted | - | - | A | - |
| 3 | JQ083494.2-ILT-Laryngovaccine | deleted | - | - | M | S | deleted | - | | A | |
| 4 | MN689093-Brazil-Virulent-MG-FarmG-P982-2016 | ----- | - | M | - | - | ----- | - | - | - | T |
| 5 | MT199135.EGY-BU Sh5.2019 | ----- | - | - | - | - | ----- | - | - | - | - |
| 6 | OM291848:ILT-Egy-kaliobia-5-2021 | ----- | - | - | - | - | ----- | - | - | - | - |
| 7 | OM291841ILT-Egy-dakahlia90-2021 | ----- | - | - | - | - | ----- | - | - | - | - |
| 6 | ILT-CK-Egy-1-2020 | ----- | - | - | - | - | ----- | - | - | - | - |
| 7 | ILT-CK-Egy-2-2020 | ----- | - | - | - | - | ----- | - | - | - | - |
| 8 | ILT-CK-Egy-3-2021 | ----- | - | - | - | - | ----- | - | - | - | - |
| 9 | ILT-CK-Egy-4-2021 | ----- | - | - | - | - | ----- | G | - | - | - |
| 10 | ILT-CK-Egy-5-2021 | ----- | - | - | - | - | ----- | - | - | - | - |

As the H9N2 is the most contributing pathogen in co infection agent that exacerbates the effects of other respiratory viruses like ILT. the histopathological examination in this study performed in detect the effect of coinfection of H9N2 and ILT in field infection of broilers. The findings of this study underscore the critical impact of co-infection with H9N2 and ILTV on broiler health, highlighting the exacerbated histopathological outcomes. The severe fibrino-hemorrhagic tracheitis observed, characterized by extensive epithelial sloughing, necrosis of mucous glands, and intranuclear eosinophilic inclusions, reflects the compounded pathogenicity of these viruses. The presence of multinucleated giant cells and significant mononuclear cell infiltration further supports the enhanced tissue damage and immunosuppression caused by co-infection. These results align with previous literature, such as (Pan *et al*., 2012; Gržinić *et al*., 2023), which demonstrated the immunosuppressive effects of H9N2, leading to increased susceptibility to secondary infections like ILTV. This study emphasizes the necessity for integrated management strategies, including robust vaccination and stringent biosecurity protocols, to mitigate the compounded effects of H9N2 and ILTV co-infections in broiler farms.

The phylogenetic analysis of ILTV isolates revealed that Egyptian field strains are closely related to the vaccine strains. While the genetic distance between field and vaccine strains is relatively small, these findings underscore the importance of continuous surveillance to monitor for potential emergence of vaccine escape variants (Perez Contreras *et al*., 2020). Moreover, while no significant mutations were observed in the ICP4 gene, genetic drift remains a concern, especially in areas with intensive poultry farming and inconsistent vaccination coverage, as this can promote the evolution of new viral strains (Kumar *et al*., 2016). The present molecular characterization of ILTV isolates revealed 97–100% nucleotide similarity with previously reported Egyptian strains, suggesting the continued circulation of endemic ILTV variants within the region. Furthermore, comparisons with CEO and TCO vaccine strains revealed high genetic similarity TCO vaccine, suggesting that the ICP4 gene remains relatively stable, thereby supporting the continued efficacy of current vaccine strains. However, the high genetic similarity between field and vaccine strains indicates a risk of vaccine-induced selection pressure, which could drive the emergence of vaccine escape mutants. This highlights the need for continuous molecular surveillance to detect any emerging mutations that could undermine current control measures (Molini *et al*., 2019). In the same way genetic characterization of ILTV strains from the (Mossad *et al*., 2022) study revealed a 97–100% similarity with circulating Egyptian strains, and they identified two major clusters: strains closely related to chicken embryo origin (CEO) vaccine strains and those related to Tissue Culture Origin (TCO) vaccine strains. Notably, non-synonymous substitutions such as R180M and S227N were found, suggesting that some field strains may have evolved from vaccine strains, contributing to the virulence observed in broiler flocks.

Co-infections involving H9N2, IBV, and ILTV not only complicate the clinical picture but also weaken the host immune response, leading to more severe disease outcomes. The presence of co-infections in more than half of the studied flocks aligns with reports from other Egyptian studies, where respiratory disease complexes were driven by viral co-circulation (Hassan *et al*., 2021). The high mortality rates observed in flocks co-infected with H9N2 and ILTV underscore the need for enhanced disease control strategies, particularly vaccination broiler chicken against ILT in endemic regions and biosecurity (Hegazy *et al*., 2019; Hassan *et al*., 2021). Previous studies have shown that co-infections can suppress the host's immune responses, delaying recovery and increasing the risk of secondary bacterial infections such as *Escherichia coli* and *Mycoplasma gallisepticum* (Jaleel *et al*., 2017; Bhat *et al*., 2022). This highlights the need for a comprehensive vaccination strategy and improved biosecurity to mitigate the impact of co-infections on poultry health.

This study underscores the critical role that mixed respiratory viral infections play in complicating disease control efforts in Egyptian broiler farms. The co-circulation of H9N2, IBV, NDV, and ILTV presents a significant challenge, as co-infections not only worsen clinical outcomes but also complicate diagnostic efforts. Furthermore, future research should investigate the role of bacterial co-infections, such as those involving *Mycoplasma gallisepticum* and *Escherichia coli*, which have been shown to exacerbate viral respiratory infections (Gowthaman *et al*., 2020; Amin *et al*., 2022). Understanding the interplay between viral and bacterial pathogens is key to developing more effective disease management strategies, which can ultimately lead to better health outcomes for poultry and reduce economic losses in the industry.

## CONCLUSIONS AND RECOMMENDATIONS

The prevalence of mixed viral infections, particularly involving H9N2, IBV, NDV, and ILTV, presents significant challenges to the Egyptian poultry industry. The high incidence of co-infections complicates both diagnosis and prevention and control measurements. While molecular characterization of ILTV in broiler chickens shows high incidence and genetic stability, continuous monitoring and biosecurity measure is essential for control ILT in broilers

as vaccination not common in this type of birds. This study underscores the importance of integrated disease management strategies, including updated vaccination protocols and enhanced biosecurity measures, to mitigate the impact of respiratory viral infections in poultry flocks. Future research should focus on the interplay between viral and bacterial co-infections to develop more broad control measures.

## ACKNOWLEDGEMENTS

The authors are grateful to the Avian and Rabbit Medicine Department at the Suez Canal University in Ismailia, Egypt, and the reference lab in Doki. For their technical assistance from all the authors.

## NOVELTY STATEMENT

Your study's comprehensive examination of respiratory viral infections in Egyptian poultry farms, particularly co-infections with Avian Influenza (H9N2), Newcastle Disease Virus (NDV), Infectious Bronchitis Virus (IBV), and Infectious Laryngotracheitis Virus, is novel. This is the first large-scale 2020–2021 Egyptian study to examine the prevalence and co-infection dynamics of these pathogens in four major governorates (Giza, Fayoum, Menya, and Menoufia). A novel aspect of ILTV isolate genetic characterisation is the stability of endemic strains and their relationship to vaccine strains in the region.

## AUTHORS' CONTRIBUTION

All authors contributed to this work. A. Orbano, M. Rady and W. Elfeil is contributor to the process and methodological processing, acquisition of data and resources, formal analysis and writing of the first draft. M. Abaza, M. Nemr, M. Rady and A. Zanaty are responsible for the supervision of sampling, methodology, and conceptualization of data and resource acquisition, formal analysis, and review of the study. M. El Demerdash contributed to conceptualization, formal analysis, validation, resources, and review. W. Elfeil and M. El Demerdash the senior supervisors of the study, directing the work, analysing the results, and writing the final manuscript. All authors carefully reviewed the final version of the manuscript and gave their approval.

### Conflict of interest

The authors have declared no conflict of interest.

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