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Molecular detection and isolation of infectious laryngotracheitis virus (ILTV) in layer farms of Waset province, Iraq

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Abstract---Infectious Laryngotracheitis (ILT) is an acute highly contagious respiratory disease of chickens. It has significant economic importance due to mortalities and the decrease in egg production. In this study, twenty-nine samples from different layer farms were collected from the outbreaks that occurred in Waset province, Iraq, at the period from November 2021 till March 2022 to detect ILTV by molecular test through real time polymerase chain reaction assay (RT-PCR) as well as isolation on embryonated chicken eggs through chorioallantoic membrane (CAM) route. The clinical signs examination of infected birds revealed extend head with expectoration of bloody mucous, gasping with weeping eye (watery eye). Gross pathological lesions examination revealed catarrhal, caseated, fibrinonecrotic, hemorrhagic tracheitis. The RT-PCR revealed amplification specific for glycoprotein G gene of ILT virus. Preparation and inoculation of tissue samples which giving positive results with RT-PCR on embryonated chicken eggs (ECE) appeared as white pock lesions on the inoculated CAM from the second passage. From these results, we can say that ILTV in Waset, one of the dangerous diseases that threaten food security and poultry wealth, and it has become imperative to pay attention to biosecurity, good management, and a safe distance between farms to avoid infections with this disease.

Keywords---infectious laryngotracheitis, ILTV, isolation, chorioallantoic membrane (CAM).

Introduction

Infectious laryngotracheitis (ILT) is a highly contagious respiratory viral disease of poultry. That has a worldwide distribution and can cause economic losses during repeat outbreaks. Caused by herpes virus with order Herpesvirales, family Herpesviridae, subfamily alphaherpesvirinae, genus Iltovirus, species Gallid herpesvirus 1 (GaHV-1)(Rojas et al., 2021), Chickens are the primary natural host, and all ages are susceptible but birds older than three weeks are more susceptible (Dufour-Zavala, 2008). Pheasant and peafowl are susceptible to ILT virus (Abdul-Aziz & Barnes, 2018). turkey sensitive in limited age (Portz et al., 2008) (Uddin et al., 2014)(Hidalgo, 2003). But Pigeons, Starlings, crows, sparrows and ducks seem to be resistant (Kaur, 2021). High morbidity and moderate mortality with respiratory signs accompanied with expectoration of bloody mucous and decrease in eggs production, Syncytial cells with intranuclear inclusion bodies was the most pathological changes of this disease (Abdul-Aziz et al., 2016)(Tran et al., 2021). In Iraq, the virus was isolated and identified in layers at the first time in 2000 in Baghdad province. (Al-Khidr et al, 2000) and also isolated and diagnosed in Baghdad and Diyala provinces by several diagnostic methods (Allawe et al., 2016) and recorded in Al-Diwaniyah province by using the molecular examination technique (Alaraji et al., 2019). Because the ILTV is enveloped, this makes it sensitive to lipolytic or organic solvents such as oxidizing agents like H₂O₂, chloroform, and ether. Replication of GaHV-1 occurs in the tracheal epithelium during the first week post infection, with ability to establish latency in the trigeminal ganglia and trachea during the lytic phase of infection (Garcia et al., 2017). The clinical signs and post mortem lesions of ILT disease are not pathogenomic, due to similar to those of other respiratory diseases (Sary et al., 2017), Therefore, laboratory methods must be used for diagnosis, Isolation of causative agent and molecular diagnostic methods, It is considered the most important methods of diagnosis, therefore, it was adopted in this study.

Materials and methods

Samples collection

Between November 2021 and March 2022, samples (trachea and larynx) were collected from twenty nine (29) layers flocks in Waset province suspected of being infected with ILTV. These flocks showed respiratory signs and expectoration of bloody mucous. The numbers of samples flocks and birds with regions shown in tables (1).

Table (1) shows numbers of sample flocks and birds with regions of samples

NO	NO. of sample flocks	NO. of sample birds	Regions
1	6	17	Kut
2	3	10	Numaniyah
3	4	11	Azizia
4	3	9	Hay
5	1	2	Jassan
6	1	2	Badra
7	4	9	Suwairah

8	2	5	Zubaidiyeh
9	2	5	Taj Al-Din
10	1	2	Zurbatiyah
11	1	2	Sheikh Saad
12	1	2	Albashayir
total	29	76	

DNA extraction and RT-PCR amplification

DNA extraction from larynx and tracheal tissue, were performed using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) based on manufacturer's instructions. Briefly, tracheas were homogenized with a mini homogenizer. The volume utilized for the DNA extraction was 200 ul. Amplification by RT- PCR by using two sets of primers, the first primer pair was used to detect region of glycoprotein G which mainly present in virulent isolates with a specific FAM labeled probe, while the second primer pair was used to detect attenuated ILT isolates (isolates that lack glycoprotein G) targeted region of J sequence downstream of glycoprotein G with a specific Quasar labeled probe.

Forward primer: 5'-CAGATCTGGCATCGCCTCAT-3'

Reverse primer: 5'-CCTGGGAACAGAACCTGAACT-3'

Probe: 5' FAM-CTAACCCGTTCCGCCGCACTCG-BHQ-3'

FAM=flurescein amidite

Quasar=Cyanine (CY5) replacement

BHQ=Black Hole Quencher

All primers were manufactured by (Alpha DNA, Montreal, Quebec) and were imported by URUK Center. The sample that showed a positive result for ILTV (virulent wild strain) was examined for three other diseases, Newcastle diseases (ND), avian influenza (AI), and infectious bronchitis (IB), in order to ensure that it was free of any other virus.

Viral isolation

According to the protocol adopted by (OIE, 2021), prepared tissue samples from positive flock. 10-11 day-old eggs were used. Eggs were inoculated into the chorioallantoic membrane with approximately 0.2 ml of supernatant (Solis, 2021) of 5 eggs and one egg was remain control positive. harvested of CAMs were according to (OIE, 2021). Presence of virus was confirmed by RT-PCR test. The second and third passage occurred in the same way, only with samples that given positive result in real time PCR test (Solis, 2021).

Results

Clinical examinations

Seventy six (76) tissue samples (trachea and larynx) were collected from twenty nine (29) layers flocks from Waset province. The lifespan of these flocks range from 10 to 30 weeks and the disease continued for about two weeks. The mortality rate reached 10% as an average, but reached 25% in some flocks with

acute form. Clinical signs were recorded; breathing patterns, conjunctivitis, and the level of depression were evaluated and scored daily for examined birds in all farms. As shown in tables (3-1) and figures (3-1) and (3-2).

Table (3-1) shows the clinical signs with number of examined birds and percentage of signs

Clinical signs		No. of cases/ Total birds	Percent
Breathing patterns	normal breathing	69/161	42.86%
	open mouth breathing	51/161	31.68%
	gasping with extended neck	23/161	14.29%
	expectoration of bloody mucous	18/161	11.18%
Conjunctivae	normal	110/161	68.32%
	swollen and partial closure of the eyes	32/161	19.88%
	complete closure of the eyes	19/161	11.80%
Depression	normal	78/161	48.45%
	mildly depression	60/161	37.27%
	severely depressed	23/161	14.29%

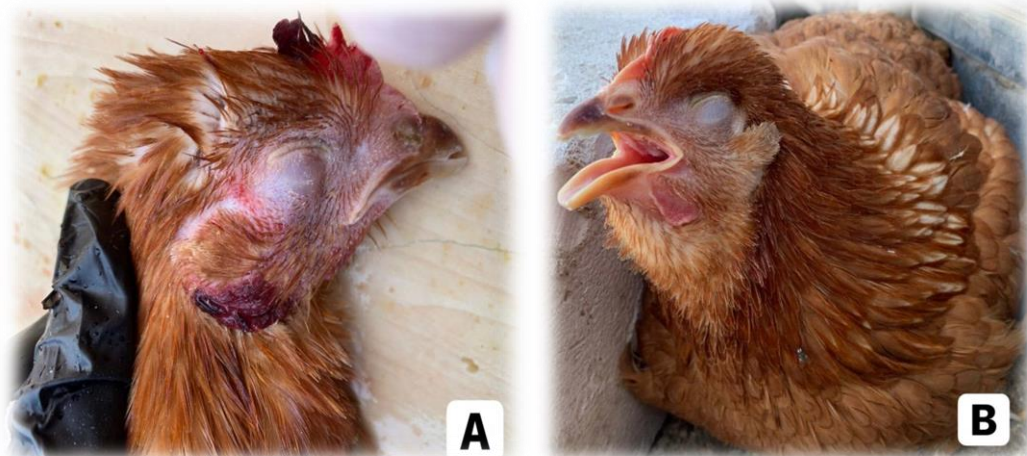


Figure 3-1: layer birds infected with infectious laryngotracheitis appear (A) present of bloody mucous, (B) difficult breathing and extend head and neck

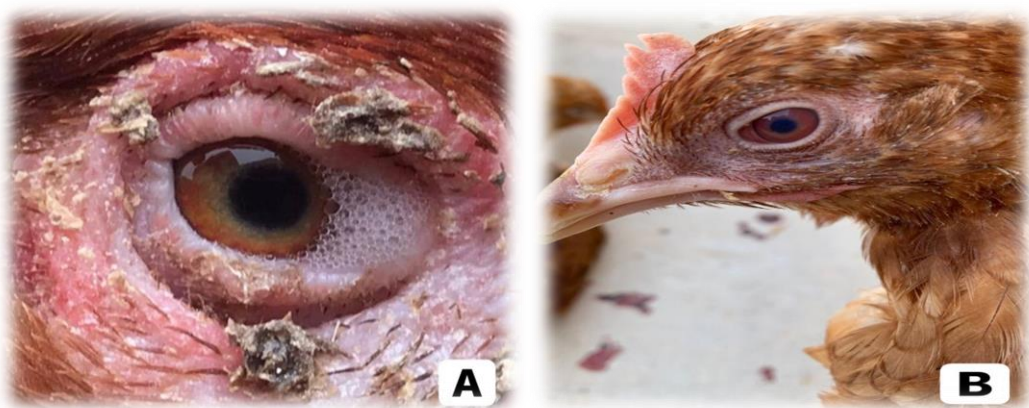


Figure 3-2: layer birds infected with infectious laryngotracheitis appear (A) weeping eye, (B) conjunctivitis

Gross pathology

The most important pathological gross lesions observed include, catarrhal tracheitis, occluded laryngeal and tracheal lumen (caseated), hemorrhagic tracheitis, fibrinonecrotic tracheitis, pulmonary congestion and Aircacculitis, as shown in table (3.2) and figures (3-3) to (3-6)

Table (3-2) Recorded gross lesion with the number of cases with observed lesion of examined birds

Gross pathology	No. of cases/total birds	Percent
Catarrhal tracheitis	55/161	34.16%
Occluded laryngeal and tracheal lumen (caseated)	75/161	46.58%
Hemorrhagic tracheitis	75/161	46.58%
Fibrinonecrotic tracheitis	61/161	37.89%
Pulmonary congestion	69/161	42.86%



Aircacculitis	58/161	36.02%
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Figure 3-3: Occluded laryngeal and tracheal lumen (caseated tracheitis)

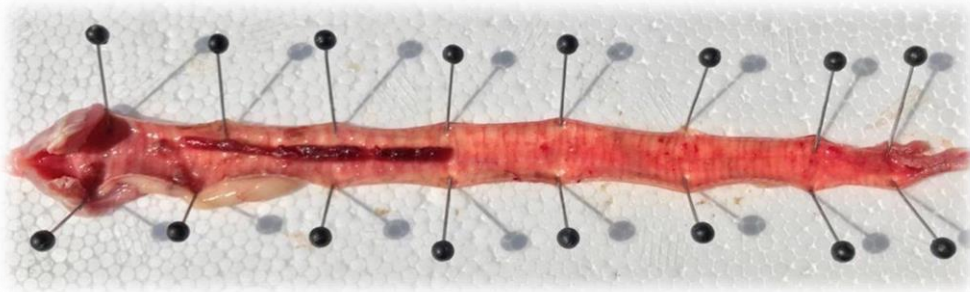


Figure 3-4: Hemorrhage with blood clot in the trachea and larynx (hemorrhagic tracheitis)



Figure 3-5: Catarrhal tracheitis.

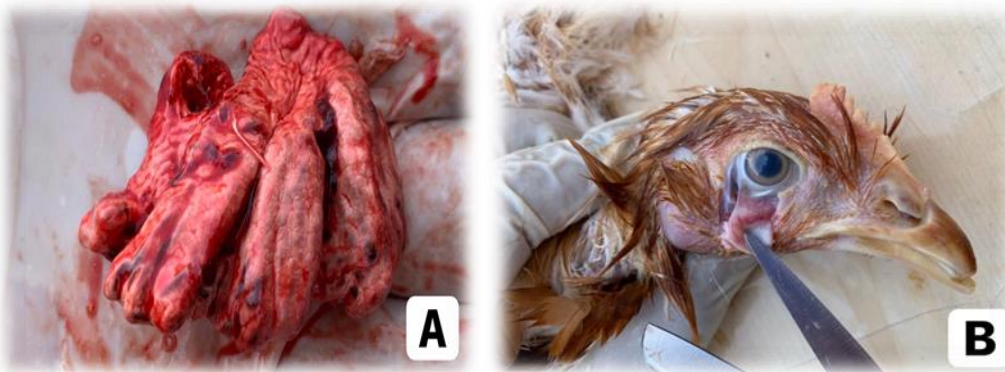


Figure 3-6: (A) edema and pulmonary congestion, (B) eyelid congestion

Detection of collected samples in real-time PCR

All twenty nine samples (farms) collected were examined by real time PCR, only twelve gave a positive result for ILTV (virulent wild strain), figure (3-7) to (3-9).

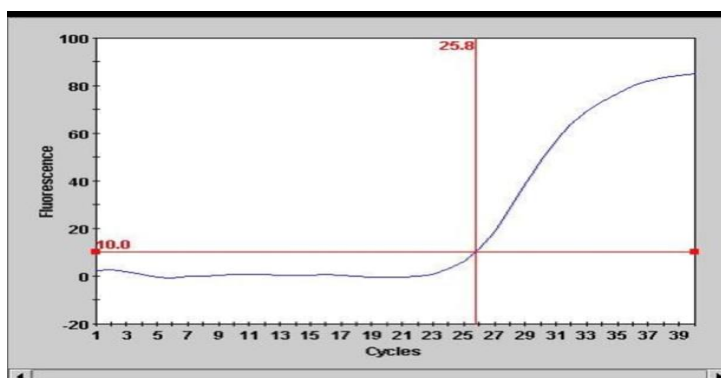


Figure (3-7): plot of real time PCR shows amplification specific for glycoprotein G gene of ILT virus

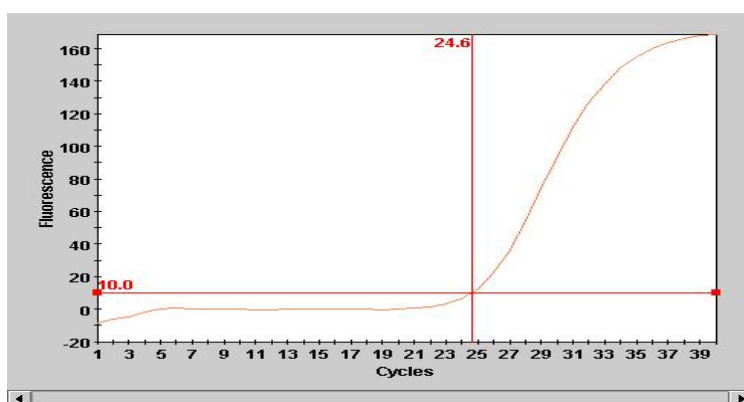


Figure (3-8): plot of real time PCR shows amplification specific for glycoprotein G gene of ILT virus

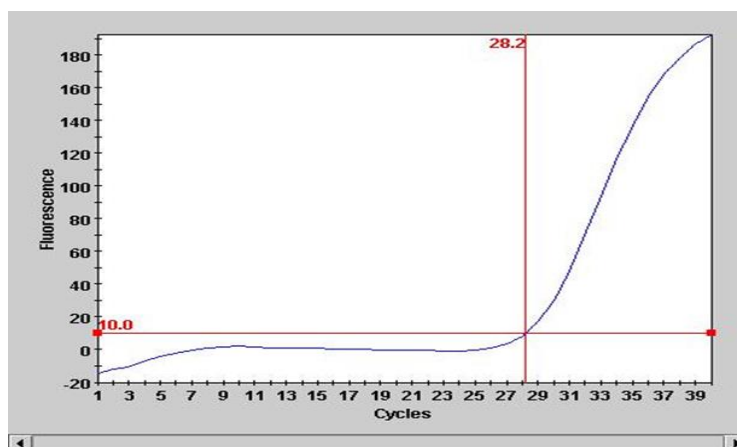


Figure (3-9): plot of real time PCR shows amplification specific for glycoprotein G gene of ILT virus

The samples that showed a positive result for ILT with Ct (25.8)(28.2) and (24.6) were examined for three other diseases, Newcastle diseases (ND), avian influenza

(AI), and infectious bronchitis (IB), in order to ensure that it was free of any other virus, where a negative result was given for all other diseases.

Results of viral isolation by egg inoculation

The first passage showed congestion, increase thickness of the membrane after 5 days post inoculation, while control CAM showed no noticeable changes. White pock lesions shown in the second passage, but the third passage showed increase thickness and hemorrhage of the CAM with clear white pock lesions which are most characteristic lesion of ILT virus after 5 days of virus inoculation as shown in the figures (3-10) and (3-11)

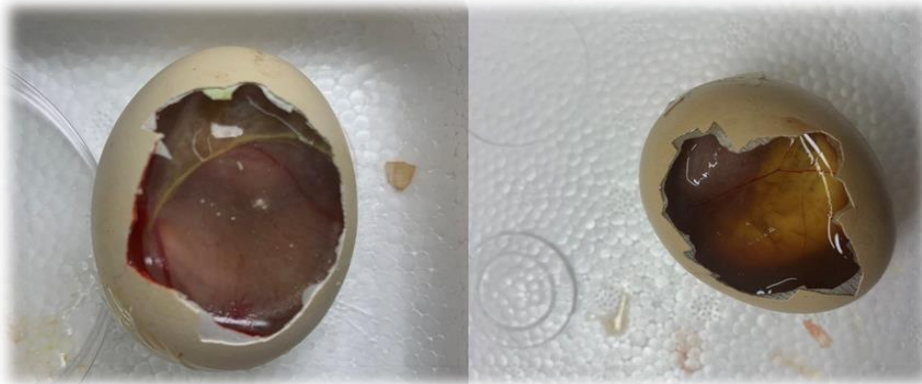


Figure (3-10) congestion and thickness of infected CAM in left compared with control CAM in right after first (1st) passage



Figure (3-11) White pock lesions in CAM after second (2nd) passage

Discussion

Infectious Laryngotracheitis is a significant respiratory disease of poultry and has a major economic effect worldwide on poultry industries (Bagust et al., 2000). This study represents the molecular detection of ILTV with isolation that caused an outbreak in commercial layer flocks in waset province, Iraq. The clinical examination of the infected chickens revealed that they showed depression, conjunctivitis and breathing patterns. These represented same clinical signs reported by (Farag & Eissa, 2021)(Hughes et al., 1991). Gross lesions of field cases revealed hemorrhagic, caseated, catarrhal, fibrinonecrotic (pseudomembrane) tracheitis. These post mortem lesions are similar to those detected by (El-Saied et al., 2021)(García et al., 2017)(Hidalgo, 2003)(Preis et al., 2013) and (Allawe et al., 2016). It is not possible to depending on clinical signs and gross lesions only for diagnosis of ILTV, so other diagnostic methods are essential. RT-PCR is an extremely adaptive ILTV recognition technique and consider the gold choice for diagnosis (Farag & Eissa, 2021). After detection of ILTV by RT-PCR, twelve samples gave positive result to virulent wild strain of ILTV, some of these samples were examined by real time RT-PCR for three other diseases, Newcastle diseases (ND), avian influenza (AI), and infectious bronchitis (IB), in order to ensure that it was free of any other virus, where a negative result was given for all other diseases, These samples that shown positive results to ILTV and negative to other viral diseases, cultivated on chorioallantoic membrane of 10-11 days-old embryonated chicken eggs, for the purpose of making sure that the pathological changes that appear on the embryo are due to the effect of ILTV only. White pock lesions shown in the second passage, but the third passage showed hemorrhage of the CAM with clear white pock lesions after 5 days of virus inoculation, which are most characteristic lesion of ILT virus. Outbreaks of ILTV strains were detected in different provinces of Iraq such as in Diwaniyah, Diyala, Baghdad and Basra which may prompt the vaccine to revert to the virulent form of ILTV (Alaraji et al., 2019) (Odisho et al., 2015)(Allawe et al., 2016). Birds with severe and very severe lesions which may be attributed to poor biosecurity measures were noted in the sampled farms showing with no adequate distance between them, high density of birds and poor ventilation as well as different vaccination protocols was used by the farms in this studied region. The increased severity of ILTV lesions may be related to extensive and unguided administration of live attenuated CEO (chicken embryo origin) and TCO (tissue culture origin) vaccines that occasionally revert to the virulent form following bird-to-bird passage in vaccinated flocks.

Conclusion

Clinical signs, gross and histopathological lesions besides the molecular detection key tools for preliminary ILT diagnosis, especially molecular detection confirm the specific diagnosis. Our findings showed variable degrees of ILTV severity in waset province. Poor biosecurity and management gave rise to recrudescence of ILT infection that emphasizes the importance of good biosecurity level to be considered.

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