Vol. 17, No. 3, Autumn, 2021, 24-33 *Original Article*
DOI: 10.22055/IVJ.2021.291306.2378
DOR: 20.1001.1.17356873.1400.17.3.3.8
Journal Homepage: http://www.ivj.ir/

Molecular identification of infectious laryngotracheitis virus in backyard and broiler chickens in Iran

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Received: 20.06.2021 Accepted: 17.08.2021

Abstract

Infectious laryngotracheitis (ILT) is a highly contagious, infectious respiratory disease that is caused by *Gallid Herpesvirus-1* (*GaHV-1*, namely ILTV) mainly affecting pullets and laying hens. The disease has not been reported in broilers and backyard chicken in Iran, previously. The present study aimed to identify ILTV strains in outbreaks with severe respiratory clinical signs that occurred in Semnan province (Iran) in broiler farms and backyard chicken farms during June 2019 and March 2020. For this purpose, specimens were investigated from twenty-eight broiler farms with severe respiratory symptoms and a mortality rate of 5.5-29% and 2 backyard flocks. The presence of ILTV was assessed by histopathology, conventional PCR targeting of ICP4 gene fragment, and sequencing analysis. Comparison of detected genotypes with strains from Iran and neighboring countries demonstrated the high homology of detected isolated with field strains from Turkey.

Keywords: Backyard chicken, Broiler, ICP4 gene sequencing, Infectious laryngotracheitis

Introduction

Infectious laryngotracheitis (ILT) is an acute, highly contagious, economically relevant infection of laying hens caused by *Gallid herpesvirus 1* (*GaHV-1*), commonly known as infectious laryngotracheitis virus (ILTV). The disease is characterized by severe dyspnea, coughing, and rales. Also, ILT may be observed as a subacute disease with nasal and ocular discharge, tracheitis, conjunctivitis, and mild rales (Hidalgo 2003; Gowthaman et al. 2020). ILTV belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and genus *Iltovirus*. It has a linear double-stranded DNA genome (155 kb in size) and can be easily transmitted by infected chicken, chickens which are latent carriers of infection, and fomites, especially in areas in which birds are intensively reared (Hidalgo 2003; Bayoumi et al. 2020). The ILT disease has been reported for the first time in Iran in 1994, when performing studies on

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avian viral infections in the country and repeated investigations and virus isolation proved the widespread and existence of the clinical forms among laying farms (Aghakhan et al. 1994).

The ILT can be caused by both field strains of ILTV and vaccine viruses. Vaccine strains have been shown to transmit immediately from vaccinated to unvaccinated birds due to insufficient attenuation of the vaccine strain. Additionally, such transmission results in vivo passage and possible reversion of vaccine virus to virulence in unvaccinated birds (Hidalgo 2003; Guy et al. 1991). ILT disease has been reported from all over the world in layer flocks and some countries in broiler chickens (KAYA and Mehmet 2018). However, there is no relevant publication regarding the ILT occurrence in broiler farms in Iran. This is the first investigation that has reported ILTV pathogenesis in the broiler farms in Iran. ICP4 (infected-cell polypeptide 4) gene detection has been applied to detect ILTV strains and for epidemiological surveys. Besides, the sequence analysis of ICP4 gene fragment has revealed outbreak-related strains of the virus in some investigations (KAYA and Mehmet 2018; Chacón and Ferreira 2009; Sadeghi et al. 2011).

The present study was carried on to investigate the occurrence of *GaHV-1* infection in broiler flocks and backyard chickens of Semnan province (Iran) suspected to ILT, and to determine the genotype of isolated viruses by ICP4 gene sequencing. Also, we compared genetic relationships of isolated strains with routine vaccine and field strains of ILTV from Iran and neighboring countries.

Materials and Methods

Case history

Between June 2019 and March 2020, twenty-eight flocks of Ross 308 broiler and two farms of backyard chickens locating in Semnan Province, Iran, were reported to have developed severe respiratory symptoms with a mortality rate of 5.5-29%. Most of the suffering birds had similar clinical signs including nasal discharge, conjunctivitis, sinusitis, coughing, moist rales, and gasping. Also, the occurrence of Newcastle disease and H9N2 Avian Influenza in farms was reported within a few past days before sampling. Most of the dead chicken from each of the farms had a distinct hemorrhage in the larynx and the tracheal mucosa. After performing the postmortem examination on infected birds, the ILT infection was suspected based on gross lesions. Thus, trachea samples and conjunctiva were aseptically taken and either frozen until they were used for molecular investigations or fixed in buffered formalin (10%) for histopathological evaluation. Procedures involving animals and their care were approved by the Animal Research Ethical Committee of Faculty of Veterinary Medicine of Semnan University, Iran (E-99/01, 25/07/2020). Procedures conformed to the criteria outlined in the Guide for the Care and Use of Laboratory Animals.

Chorioallantoic membrane (CAM) inoculation of ILTV in chicken embryonated eggs

Ten tracheal samples from each broiler flock were pooled and assumed as one sample. finally, 28 pooled tracheal samples of diseased birds from 28 broiler flocks were processed for virus isolation. Regarding to backyard chicken farms, 5 tracheal samples, because of low mortality rate, were pooled and assumed as on sample and finally 2 samples from backyard chicken farms were processed for virus isolation. Briefly, a 20% solution of the tracheal samples in PBS was prepared by mincing samples using a sterilized mortar pestle plus liquid nitrogen. Homogenized samples were centrifuged at 13,000 rpm for 20 min at 4°C. Then, the supernatant was passed through a 0.45 μm filter (Millipore) and incubated with an antibiotic solution (streptomycin 200 μg/ml and penicillin 200

IU/ml) for 1 hr at 37°C. Finally, prepared supernatants were inoculated into the CAM of 12-13-day old SPF eggs as previously described in Magouz et al. (2018) by the artificial air sac route for up to three blind passages. The eggs were incubated at 37°C for a week and daily examined for any abnormalities.

Molecular identification of ILT based on partial sequencing of ICP4

Genomic DNA extraction was carried out on 30 tracheal tissue samples showing clinical signs of ILT by using a commercial extraction kit (DynaBio™ Blood/Tissue DNA Extraction Mini Kit, Takapouzist, Iran). Then, extracted genomes were subjected to PCR for amplification of a 688 bp fragment of the ICP4 gene belonging to the ILT virus. Identification and amplification of target sequence were carried out using previously published primers: (forward ICP4-F: 5′-ACTGATAGCTTTTCGTACAGCACG -3′ and reverse ICP4-R: 5′-CATCGGGACATTCTCCAGGTAGCA –3) (Chacón and Ferreira 2009).

The nucleotide sequence of the amplified fragments was analyzed with an ABI 3730XL DNA Analyzer according to an automated Sanger dideoxy fluorescent nucleotide method. Then, the BLAST software was applied to determine the homology of the amplified fragment to DNA sequences existing in GenBank, and the phylogenetic tree was constructed by the neighbor-joining model in MEGA-X.

Histopathology of trachea specimens

All 20 positive trachea samples in molecular identification from backyard chicken and broiler farms, were routinely prepared for histopathologic evaluations via paraffin embedding and cutting. Then, procedures including deparaffinization, dehydration, and staining with hematoxylin and eosin (H&E) were carried out according to the protocol described by Bancroft and Gamble (2008).

Results

All details about positive broiler and backyard chicken farms with ILTV have been mentioned in table 1.

Gross pathology and histopathology findings in trachea and larynx

At necropsy, the most lesion was found in the upper larynx and trachea, such as clotted blood and hemorrhagic mucofibrinous. The lower part of the trachea had fewer lesions than the upper part. In some cases, small hemorrhages with swelling in the eyelid and nasal cavity of birds were seen (Figure 1). In histopathology sections, marked edema, inflammatory cells, lymphocyte infiltration were found in tunica propia and submucosa layers in the trachea, the dence hemorrhagic and inflammation exudate were significant on the surface of necrotic mocusa layer (Figure 2). Edema, elastic fiber, and hyperemia and hemorrhage around small vessels in sub mocusa layer were significant and intranuclear inclusion bodies with changes the nuclei described herpesviridea (Figure 2).

ILTV propagation on embryonic egg

16 out of 28 (broiler farms) suspensions inoculated to embryonic eggs showed evidence regarding viral growth such as stunting and congestion. Pock lesions in the chorioallantoic membrane were observed in 11 samples on the first egg passage except for 2 samples which showed pock lesion only after two and three passages, respectively (Figure 3). The remaining inoculated eggs were either negative even after three passages or showed bacterial or fungal infections after inoculation.

Table 1: Detailed data regarding the ILTV infected flocks in Semnan province

Sample number	Farm number	Location	Type	Age /Days	MOR.[c] (%)	VAC. HIS[d]
1	1	Sorkhe	BRLR[a]	46	21	AI/ ND/ IBV/ IBD
2	2	Sorkhe	BRLR	48	18.5	AI/ ND/ IBV/ IBD
3	3*	Momenabad	BRLR	49	8	AI/ ND/ IBV/ IBD
4	3*	Momenabad	BRLR	45	17.5	AI/ ND/ IBV/ IBD
5	4*	Biabanak	BRLR	39	22	AI/ ND/ IBV/ IBD
6	4*	Biabanak	BRLR	42	16	AI/ ND/ IBV/ IBD
7	5*	Ala	BRLR	51	5.5	AI/ ND/ IBV/ IBD
8	5*	Ala	BRLR	46	16.5	AI/ ND/ IBV/ IBD
9	6	Lasjerd	BRLR	38	24	AI/ ND/ IBV/ IBD
10	7*	Sorkhe	BRLR	43	15.5	AI/ ND/ IBV/ IBD
11	7*	Sorkhe	BRLR	48	18	AI/ ND/ IBV/ IBD
12	8	Ahowan	BRLR	45	6.5	AI/ ND/ IBV/ IBD
13	9	Aftar	BRLR	47	11	AI/ ND/ IBV/ IBD
14	10	Ala	BRLR	41	19	AI/ ND/ IBV/ IBD
15	11*	Aftar	BRLR	44	21	AI/ ND/ IBV/ IBD
16	11*	Aftar	BRLR	46	29	AI/ ND/ IBV/ IBD
17	11*	Aftar	BRLR	39	17	AI/ ND/ IBV/ IBD
18	12	Sorkhe	BRLR	43	19	AI/ ND/ IBV/ IBD
+19	13	Ala	BYD[b]	150	1	AI/ND
#20	14	Biabanak	BYD	163	0.8	AI/ND

[a]: Broiler/ [b]: Backyard Chicken/ [c]: Mortality Rate from 8 Days of Onset of ILT Clinical Signs / [d]: Vaccination History

AI: Avian Influenza/ ND: Newcastle Disease/ IBV: Infectious Bronchitis Virus/ IBD: Infectious Bursal Disease

*: broiler farms with recurrent ILT incidence in continual rearing periods

+: flock size: 4500, #: flock size: 1200

ILTV detection by ICP4-based conventional PCR

ILT DNA was detected in 18 out of 28 (64.2%) tracheal specimens of broiler farms, in 2 out of 2 backyard chicken farm and in vaccine strains as positive control. Negative specimens were evaluated twice to confirm the results. All of the ILT-positive clinical specimens were obtained from broiler and backyard chicken farms that had not been vaccinated against ILT, previously (Figure 4).

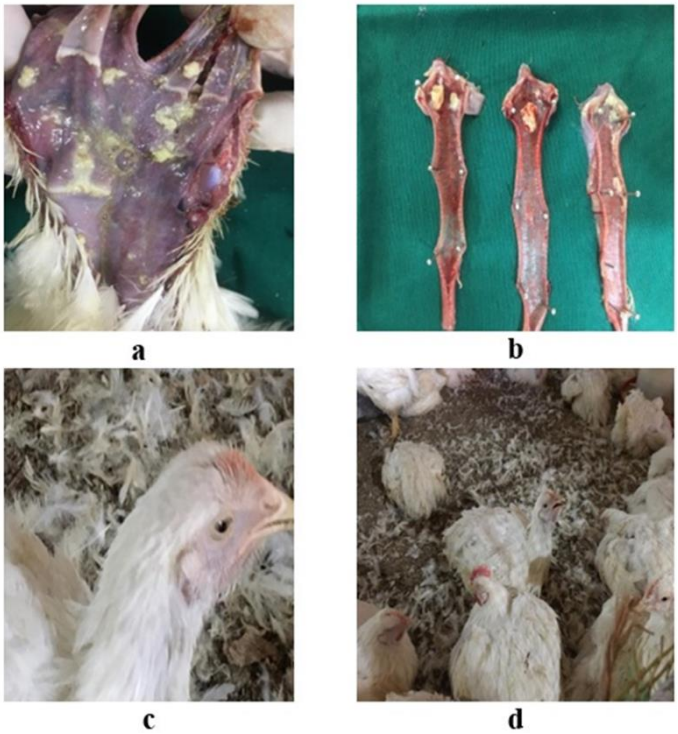

Figure 1. Significant suppurative masses in trachea (a & b). Hemorrhages in the mucous membrane or a small amount of mucus mixed with dotted blood and restricted entirely to the upper larynx (c). Head swelling with sinusitis (c) typical gasping was shown in broiler flocks (d).

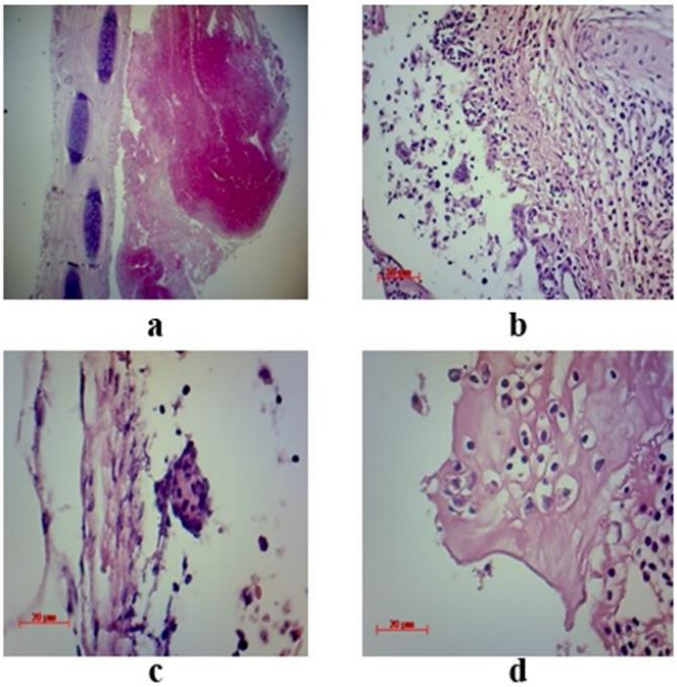

Figure 2. Dense hemorrhagic and infilamative exudate significant in the upper of necrotic mocusal layer (a). The dense cellular infiltration in both tunica propria and submucosa in the larynx and trachea (b). The sancetial cell is shown in the upper layer of mocusa with same cytoplasm and many nuclei (c). The appearance and size of the intranuclear inclusion bodies as well as the changes in the nuclei bore a close resemblance to those described in *herpesviridae* (d).

Figure 3. Isolation and identification of field strains of ILTV in the present study representing pock lesions in the chorioallantoic membrane (CAM) in embryonic eggs.

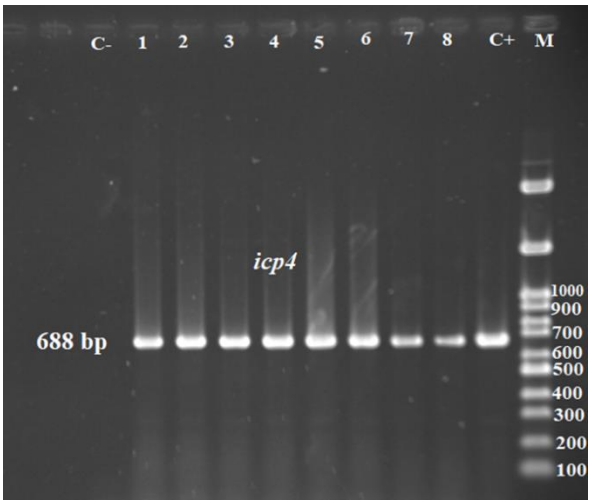

Figure 4. Gel electrophoresis of PCR products for detection of ILTV strains based on ICP4 gene fragment. Lane M: 100 bp marker. Lanes C+ and C-: positive (vaccine strain) and negative controls (*E. coli* genome). Lanes 1-8: ILTV detected DNA in trachea specimens.

Phylogenetic analysis of the amplified ICP4 gene fragments

The sequence alignment was used to generate a phylogenetic tree via the neighbor-joining method with the MEGA-X Software package. A consensus phylogenetic tree, including strains from Iran and neighboring countries, was built (Figure 5). The obtained sequence was deposited in GenBank with accession numbers: MT440055, MT440056, MT440057, MT440077, MT440078 for broiler farms and MT440058, MT525968 for backyard chicken farms.

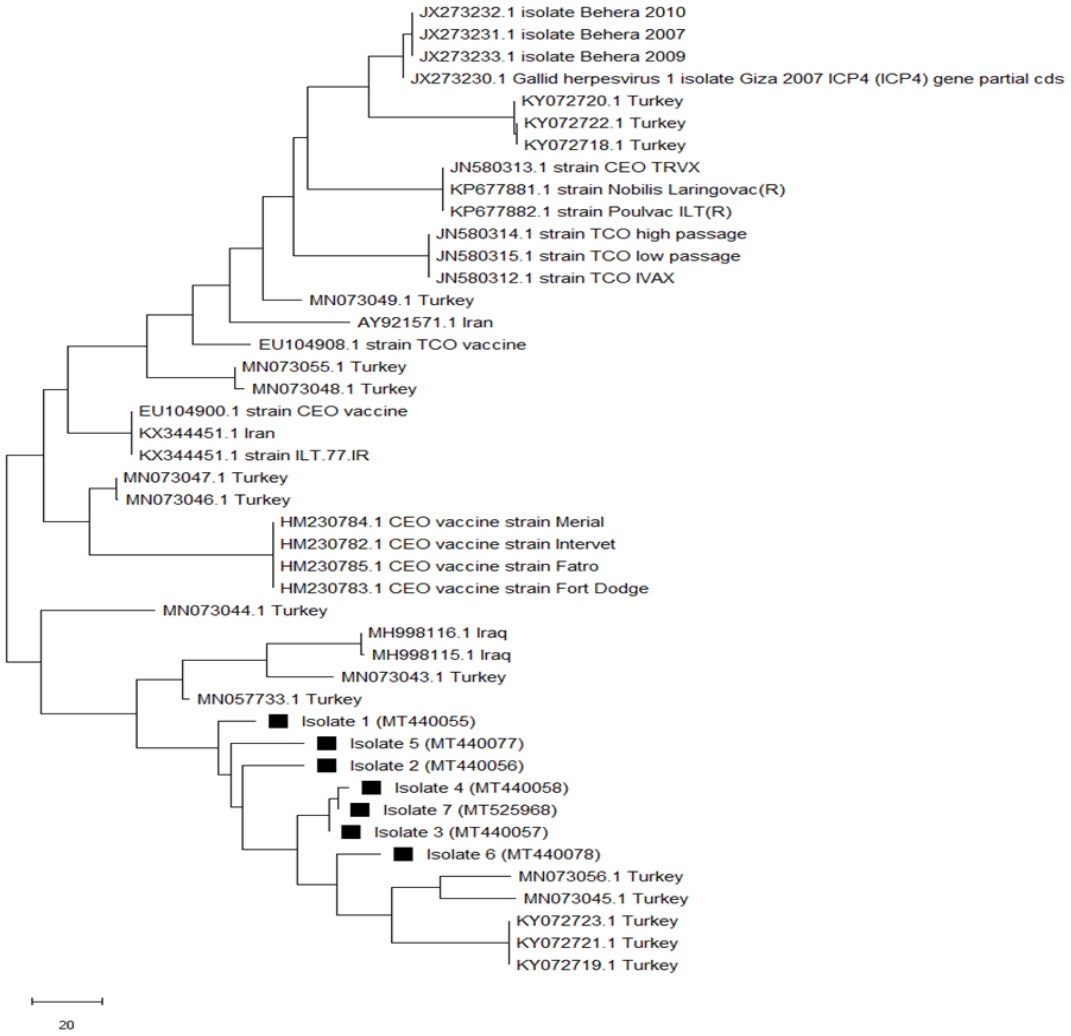

Figure 5. Neighbour-Joining phylogenetic tree based on the ICP4 gene sequence of the infectious laryngotracheitis virus. *Note:* the marked names with ■ representing the detected strains in this study and CEO & TCO represent vaccine strains.

The obtained phylogeny tree shows that all of the detected ILTV strains in broilers and backyard chickens from Semnan region fitted together in one cluster of the phylogenetic tree, with heterogeneity from routine vaccine strains and some other field strains, for instance from Iran and Turkey. Some field strains of ILTV circulating in neighboring countries including Turkey and Iraq belong to the same clade as the detected strains in the present investigation showing higher genetic homology with our isolates.

The other main clades of the phylogenetic tree include the field and vaccine strains of ILTV from the USA, Australia, Korea, and Egypt.

Discussion

Infectious laryngotracheitis virus has usually been considered an acute pathogen in laying pullets while its role as a primary agent has often been lessened in broilers. Birds may be infected at any age but clinical symptoms are observed in birds older than 4 weeks (Gowthaman et al. 2020). Nevertheless, unscientific communications from farmers and veterinarians started reporting the rising impact of ILTV in broilers. Unfortunately, the limited availability of published scientific epidemiology documentation regarding ILT in broilers prevents any convincing data. Also, the majority of published data arise

from a few geographic regions and are poorly shared through time, while a concise data report would be necessary for the understanding of ILT epidemiology and its evolvement in broilers.

The record of ILT in broilers backs to 1925 by May and Tittsler who described an outbreak on a farm in Rhode Island in 1923 (May and Tittsler 1952). Then, in subsequent years there have been various reports of the infection in broilers from many parts of the world (Hidalgo 2003). ILTV is known to be endemic in Iran and causes severe economic losses and mortality in the poultry industry. To the best of our knowledge, in 1994, Aghakhan et al. reported the presence of ILTV in Iran without further details on the virus strains (Aghakhan et al. 1994). There are 2 studies regarding ILTV infection in broilers in Iran (Ghalyanchi et al. 2020; Razmyar et al. 2021). Ghalianchi et al. investigated the seroprevalence of the ILTV in four provinces by ELISA method and found 13 % of seropositivity in broiler flocks (Ghalyanchi et al. 2020). Besides, Razmyar et al. reported co-infection of ILTV and Newcastle disease accompanied with Salmonella Enteritidis occurred in a broiler flock in the western region of Iran (Razmyar et al. 2021). The present investigation is comprehensive research reporting the detection, pathogenicity, and sequencing of ILTV strains in Ross 308 broiler flocks and backyard chicken flocks in Iran for the first time. The examined broiler farms were positive for ILTV at 5-7 weeks of age, as determined by isolation of the virus in embryonic eggs and conventional PCR method, confirming previous findings in broilers at similar statues including age of infection (Hidalgo 2003; KAYA and Mehmet 2018; Can-Sahna et al. 2020).

Although it is a fact that our results are absolutely preliminary, they already provide valuable new information regarding the pathogenesis of ILTV in broilers and backyard chickens. Phylogenetic analysis of the ICP4 gene of the detected viruses proved the very high homology between strains currently being detected not only in laying pullets but also in broilers (KAYA and Mehmet. 2018; Can-Sahna et al. 2020).

As shown in Figure 5, the sequence obtained from the detected ILT viruses were closely related to that of the reference strains MN073056.1; MN073045.1; KY072723.1; KY072721.1 and KY072719.1 which have been identified from pullets and broilers in Turkey, previously. KY072723.1, KY072721.1, and KY072719.1 are ILTV strains considered to be primary pathogens associated with ILT in broiler farms reported by Kaya and AKAN to cause significant infectious laryngotracheitis lesions in meat-type chickens (KAYA and Mehmet). The isolated ILTV strains in our investigation were also closely related to other virus strains from Turkey and Iraq which were known to cause respiratory and visceral lesions in layer hens with mortality described previously by Can-Sahna et al (Can-Sahna et al. 2020). A notable result in our study is the more genetic diversity of detected strains with ILTV strains previously identified in Iran (AY921571; KX344451) compared to Turkey and Iraq strains and this probably represents the same origin of our strains with the strains from Turkey and Iraq. For the prevention of ILT in layer hens, two types of live attenuated vaccines (chicken embryo-origin, CEO & tissue culture origin, TCO) has been widely used (Hidalgo 2003). In previous publications, it has been demonstrated that attenuated vaccines can be associated with some complications including reversion of virulence, the capacity to infect non-immune chickens, and generation of latently infected reservoir hens (Sadeghi et al. 2011). Also, ILTV isolates related to the CEO vaccine have been detected from severe infectious laryngotracheitis outbreaks, frequently (Hidalgo 2003; Guy et al. 1991). None of

the ILTV isolates in the present study were genetically similar to vaccine strains used in Iran (Figure 5) indicating the prevalence of wild strains of ILTV in broiler farms.

Histopathological analysis revealed compatible lesions with ILT such as epithelial necrosis and serofibrinous and serohemorrhagic exudates in the trachea of chickens from all broiler farms (Figure 2). Our findings are in agreement with previous reports by Bagust et al. who demonstrated that specific lesions of ILT are observed between 3-9 days post-natural and experimental ITLV infections (Kaboudi et al. 2016; Bagust et al. 2000). Kaboudi et al. concluded that the severity of observed lesions may be variable based on the examined tissues because of the ILTV strain affinity for various tissues (Kaboudi et al. 2016). Also, many strains of ILTV exist that deserve a high affinity for the trachea and very low tropism for the vice versa and conjunctiva (Timurkaan et al. 2003). Additionally, embryonated eggs inoculated with the ILTV suspected specimens revealed characteristic pock lesions on the chorioallantoic membrane in eleven specimens on the first egg passage while two specimens needed further blind passages in embryonated eggs. These results regarding the rate of detection in eggs were in parallel with previous investigations (Magouz et al. 2018; Crespo et al. 2007).

Considering that there is genetic similarity between reference strains (from layers and broilers) and field strains of ILTV in the present study, this knowledge should be taken into account in the selection of the applied vaccination program and probable virus transmission ways from laying hens to broilers. However, it must be stressed that ILTV strains in broilers may be vaccine strains; thus, if the used vaccines in laying hens are pathogenic for broilers, a proper and preventive schedule regarding ILT in broilers must be considered. Subsequently, molecular epidemiological analysis of field strains is a robust tool to determine the current ILTV condition towards establishing an urgent disease control policy.

In conclusion, virulent ILTV field strains were detected from Semnan province and display close phylogenetic homology to field strains circulating in Turkey. The preliminary results of this study allowed us to report the first comprehensive research and identification of ILTV field strain in broiler and backyard chicken farms in the presence of clinical signs. This evidence stresses once more the need for a more systematic ILTV monitoring and surveillance investigations in layers, broilers and backyard chickens which will be essential to obtain a better understanding of virus epidemiology and to rationally plan effective control strategies concerning ILT in the poultry industry.

Acknowledgements

Authors express their willing's to the Head of Faculty of Veterinary Medicine, Semnan University for providing facilities for the present study.

Conflict of interest

The authors disclose any financial and personal relationships with other people or organizations that might inappropriately influence or bias this work.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Received: 20.06.2021
Accepted: 17.08.2021

DOI: 10.22055/IVJ.2021.291306.2378
DOR: 20.1001.1.17356873.1400.17.3.3.8

تشخیص ملکولی ویروس لارنگوتراکئیت عفونی در جوجه‌های بومی و گوشتی ایران

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دریافت: ۱۴۰۰/۳/۳۰ پذیرش: ۱۴۰۰/۵/۲۶

چکیده

لارنگوتراکئیت عفونی یکی از بیماری‌های بسیار مسری در دستگاه تنفس طیور است که توسط هرپس ویروس‌های گروه ۱ (*GaHV-1*) مربوط به پرندگان ایجاد شده و عمدتا پولت‌ها و گله‌های تخمگذار را درگیر می‌نماید در حالی که در گله‌های گوشتی و بومی ایران تاکنون گزارش نشده است. هدف از تحقیق حاضر تشخیص و تعیین هویت جدایه‌های ویروس لارنگوتراکئیت عفونی در مواردی از همه‌گیری علایم شدید تنفسی در گله‌های گوشتی و بومی استان سمنان در بازه زمانی ژانویه ۲۰۱۹ تا مارس ۲۰۲۰ می‌باشد. در این راستا نمونه‌های مربوط به ۲۲ مزرعه مرغ گوشتی با علایم بالینی شدید تنفسی که تلفات ۲۹ – ۵/۵ درصد داشته‌اند و همچنین دو گله مرغ بومی با علائم مشابه مورد بررسی قرار گرفتند. حضور ویرس لارنگوتراکئیت عفونی طیور به عنوان عامل ایجاد بیماری با استفاده از روش‌های هیستوپاتولوژی، ردیابی ژن ICPI و تعیین توالی نوکلئوتیدی ژن یاد شده مورد ارزیابی قرار گرفت. بررسی مقایسه‌ای توالی‌های به دست آمده با جدایه‌های کشور ایران و کشورهای همسایه نشان دهنده مشابهت بالای سویه‌های جدا شده در این تحقیق با سویه‌های جدا شده از کشور ترکیه می‌باشد.

کلمات کلیدی: مرغ‌های بومی، مرغ گوشتی، توالی ژن ICPI، لارنگوتراکئیت عفونی طیور

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