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Investigation of Infectious Laryngotracheitis Virus in Broiler Flocks in Sulaymaniyah Province, Iraq

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A B S T R A C T

Infectious laryngotracheitis (ILT) is an acute contagious upper respiratory tract infection of chickens and other birds, caused by Gallid herpesvirus1 (GaHV-1), which has economic importance in the poultry industry. There was no scientific data about the incidence of the disease in broiler farms in Sulaymaniyah province/Iraq. Therefore, this study aimed to investigate ILTV infection in broiler farms in that region. Clinically infected birds from 89 broiler flocks that had respiratory distress, coughing, gasping, tracheal rales, nasal ocular discharge, and congested trachea with purulent exudate, hemorrhagic tracheitis with/or without necrotic changes were investigated. The DNA was extracted from the pooled samples, including tracheal secretion, trachea, and lung tissue. Primers specific to the thymidine kinase gene (tdk) of ILTV- were used in PCR to detect the virus. A phylogenic tree was generated to track the virus's origin. The study revealed that the rate of infection with ILTV among broiler farms was 2.2% (2/89) in the region. The sequencing analysis showed that the ILTV isolated in the area was closely related to the reported strains in the United States and Brazil (MN643591.1 and S83714.1); and had a sequence identity of 98.27% to the taxon JQ217378.1. In conclusion, the study reported that one of the causes of the respiratory viral infection in broiler flocks even at younger ages was related to ILTV. Partially sequenced tdk gene of the virus showed that the circulated serovar in the region had some nucleotides and amino acids differences with the worldwide reported serovars. This should be taken into consideration in the poultry industry by doing further investigation.

Keywords: Infectious laryngotracheitis, ILTV, Broiler, Sulaimaniyah, Phylogenic, Gallid herpesvirus1

INTRODUCTION

T

he infectious laryngotracheitis (ILT) is an acute, highly contagious upper-respiratory tract infectious disease in poultry; and it was firstly observed in the United States in 1925 (1). The disease is caused by infectious laryngotracheitis virus (ILTV), also called Gallid herpesvirus-1(GaHV-1), which belongs to the family Herpesviridae, subfamily Alphaherpesvirinae and genus Iltovirus (2). The ILTV genome contains 150-155 Kb linear double-stranded DNA encoding a unique long (UL), two unique short (US) and two inverted repeats (IR) sequences (2-4). Despite, the susceptibility of older chickens to the virus, ILT has been reported in broiler chicks as early as three weeks of age (5, 6). It was mostly found in layer flocks, and recently appeared to be one of the most serious infectious diseases in broilers Farms (5-7). Other birds, including peafowl, pheasant and turkeys can also be infected with the virus. While other birds, such as ducks, sparrows, pigeons, starlings and crows seem to have

resistance to the virus (2, 8). This virus is easily transmitted horizontally among infected birds and chickens, which are latent carriers of infections (4, 9, 10). The severity of the ILTV infection is affected by the virus's virulence, stress factors, co-infections with other viruses, flock density, bird immunological status, and chicken age (11). This disease causes clinical signs similar to other respiratory viral infections, these signs include conjunctivitis, respiratory distress, gasping, sinusitis, coughing, extending of necks, asphyxia and tracheitis (12-14). Infection with this virus may be observed as a sub-acute disease with nasal and ocular discharge, tracheitis, conjunctivitis, and mild rales (2, 6, 13). There are two major forms of ILTV infection in chickens, these are the epizootic form (severely acute) and the mild form (2). The epizootic form is characterized by respiratory distress, sneezing, expectoration of blood-mixed mucus, severe hemorrhagic tracheitis and conjunctivitis with a high mortality rate (ranging from 5% to 70%). While the mild ILTV infection form is characterized by mild to moderate catarrhal tracheitis, sinusitis, conjunctivitis and a low morbidity and mortality rates, which occasionally ranges between 0.1% to 0.2% (2, 15).

The postmortem lesions were restricted to the upper respiratory tract, primarily the trachea, which showed mucoid and hemorrhagic to necrotic tracheitis, and in some cases caseous diphtheritic membrane adherent to the larynx and trachea was also observed (6, 10). There is no any effective specific treatment for ILTV infectious, biosecurity and/or vaccination programs are the most effective methods to control the disease (6, 16). Previously an outbreak of the disease was reported in layers in some parts of Iraq, including Al-Sawara city (17) and Al-Diwaniyah province(18). Experimentally, it was proven that the ILTV can infect broiler chickens at all ages (12). Despite having the highest rate of respiratory viral infections in the region, to the best of our knowledge, there is no any study about ILT infection, especially, in broiler farms in Sulaimaniyah province. Therefore, the study aimed to investigate the ILTV infection in broiler farms in Sulaimaniyah province, using polymerase chain reaction (PCR) followed by sequencing the PCR products to confirm presence of the virus and to track its origin.

MATERIALS AND METHODS

Ethics

The study plan was approved by the Ethics Committee (No. 11.2021) and conducted according to the relevant guidelines and regulations at the college of Veterinary Medicine, University of Sulaimani.

Clinical Examination, Necropsy and Sampling

Eighty-nine broiler farms from different regions in Sulaimaniyah governorate were investigated during the period from December 2021 to April 2022. Birds aged between 10-50 days with common respiratory signs were selected and subjected to clinical and postmortem examination. Samples, including respiratory secretions, trachea and lung tissues were aseptically collected from 3-5 sick birds per flock. Approximately 5-10 mg of pooled samples from each flock were kept in a 1.5 ml Eppendorf tube and transported to the college of Veterinary Medicine-Postgraduate Laboratory, University of Sulaimani in cold boxes for further investigation.

Molecular Detection of *L. plantarum*

DNA extraction

Total DNA was extracted from the pooled samples using a commercial Addprep Viral Nucleic Acid Extraction Kit (Add Bio, Inc., Korea) according to the manufacturer's instructions. The extracted DNA from live attenuated-Serva vaccine strain of ILTV of chicken embryo origin (Nobilis® ILT, Intervet) was used as a positive control**.**

Polymerase chain reaction

Primers specific to the ILTV were used to amplify 673 bps of the tdk-gene using PCR. Briefly, 20 µl PCR reaction was prepared by mixing 1 µL (10 pmole/1 µL) of each forward and reverse primer (5`-AGGTTGCCGTCTATACTTAGC-'3 and 5`-GCA ATAGCGTCTGGTCGATTG-'3, respectively) (19), 10 µL of Add Taq Mastermix, 5 µL of the sample DNA, and the volume was completed to 20 µL by adding 3 µL of Nuclease-free water. The amplification program was: 95 °C for 5 min for the initial denaturation step. Then, 95 °C for 30 sec for denaturation, 61 °C for 30 sec for annealing, and 72 °C for 30 sec for the extension step, were repeated for 35 cycles. The final extension step was done at 72 °C for 5 min using thermo-cycler (Techne® Prime, UK). Then, the bands of the amplified PCR product were visualized on 1.5% agarose gel using UV Transilluminator gel image documentation system (Ingenius, USA) (Supplementary data).

DNA sequencing and phylogenetic tree analysis

One amplicon was sequenced using Sanger DNA sequencer (Macrogen Co., Korea). After a proper removal of incorrect peaks especially at the start and end of the DNA sequence, the obtained sequence was blasted against other reported sequences deposited on GenBank to validate the sequence identity. In addition, the amino acids sequences and their corresponding codons were predicted using ExPASy Server, Clustal Omega (Multiple Sequence Alignment), NCBI nucleotide blast and MEGA-X software.

Statistical Analysis

All data were analyzed using Cross-tabulation (SPSS). Chi-square was used to find an association between the

variables. *P*-Value less than 0.05 was considered statistically significant.

RESULTS

The investigated birds had common respiratory clinical signs, including respiratory distress, coughing, gasping, tracheal rales and nasal ocular discharge. Their postmortem lesions characterized by presence of congested trachea with purulent exudate, and hemorrhagic tracheitis with/or without necrotic changes.

The results showed that the rate of infection with ILTV in Sulaimaniyah province was 2.2% (2/89). The infection was reported in younger ages (15-25 days) (Table 1). The infected chickens had a co-infection with infectious bronchitis (unpublished data). The findings also showed that there was no associations between the farm capacity, scaled-time scheduled and incidence of the disease (Table 2) as the disease was reported during January and March (Table-3). Furthermore, no association was seen between the vaccination of the chicks with other vaccine types, including IBV, ND and IV, and the rate of infection by ILTV (Table 4).

Table 1. Frequency and percentage, and associations between ILTV infection and age range groups

		Frequency and percentage according to the age groups					
Pathogen	**Result**	**<15 days**	**Between 15-25 days**	**Between 26-35 days**	**>35 days**	**Total**	***P*-value**
	Positive	0(0.0%)	2(100%)	0(0.0%)	0(0.0%)	2(2.2%)	0.489
	Total	0.0%	2.2%	0.0%	0.0%		
ILTV	Negative	15(17.2%)	47(54.0%)	19(21.8%)	6(6.9%)	87(97.8%)	
	Total	16.9%	52.8%	21.3%	6.7%		
	Total	15(16.9%)	49(55.1%)	19(21.3%)	6(6.7%)	89(100%)	

Table 2. Frequency and percentage, and associations between ILTV infection and farm capacity

		Farm capacity (number of chickens/farm)				
Pathogen	**Result**	**<12000**	**Between 12000-14000**	**>15000**	**Total**	***P*-value**
	Positive	1(50.0%)	1(50.0%)	0(0.0%)	2(2.2%)	0.192
	Total	1.1%	1.1%	0.0%		
ILTV	Negative	10(11.5%)	35(40.2%)	42(48.3%)	87(97.8%)	
	Total	11.2%	39.3%	47.2%		
	Total	11(12.4)	36(40.4%)	42(47.2%)	100%	

Table 3. Scaled-time scheduled and the incidence of the disease

		Date of collection (Month)						
Pathogen	**Result**	**November**	**December**	**January**	**February**	**March**	**Total**	***P*-value**
	Positive	0(0.0%)	0(0.0%)	1(50.0%)	1(50.0%)	0(0.0%)	2(2.2%)	0.520
	Total	0.0%	0.0%	1.1%	1.1%	0.0%		
ILTV	Negative	5(5.7%)	31(35.6%)	13(14.9%)	28(32.3%)	10(11.5%)	87(97.8%)	
	Total	5.6%	34.8%	14.6%	31.6%	11.2%		
	Total	5(5.6%)	31(34.8%)	14(15.7%)	29(32.7%)	10(11.2%)	89(100%)	

Table 4. The correlation between vaccination of birds with other vaccines and the incidence of the disease

		Vaccine status (IBV, ND and IV)			
Pathogen	**Result**	**Yes**	**No**	**Total**	***P*-value**
	Positive	1(50.0%)	1(50.0%)	2(2.2%)	0.430
	Total	1.1%	1.1%		
ILTV	Negative	65(74.7%)	22(25.3%)	87(97.8%)	
	Total	73.0%	24.7%		
	Total	66(74.2%)	23(25.8%)	89(100%)	

Nucleotides and Amino Acid Sequences with the Phylogenetic Tree Analysis

The partially sequenced DNA of the tdk-gene of ILTV was successfully deposited under a specified accession number (OP038919) on GenBank/ NCBI. The sequence of this study was found to have 100%-98.27% homology to other taxons. It was 100% similar to MN643591.1 and S83714.1, which were isolated from frozen trachea tissue of Gallus gallus in Brazil and chicken in the USA, respectively. It had about 99.71%-98.27 sequence identity to each of EU423887.1, JN542536.1, KC248170.1 and JQ217378.1.

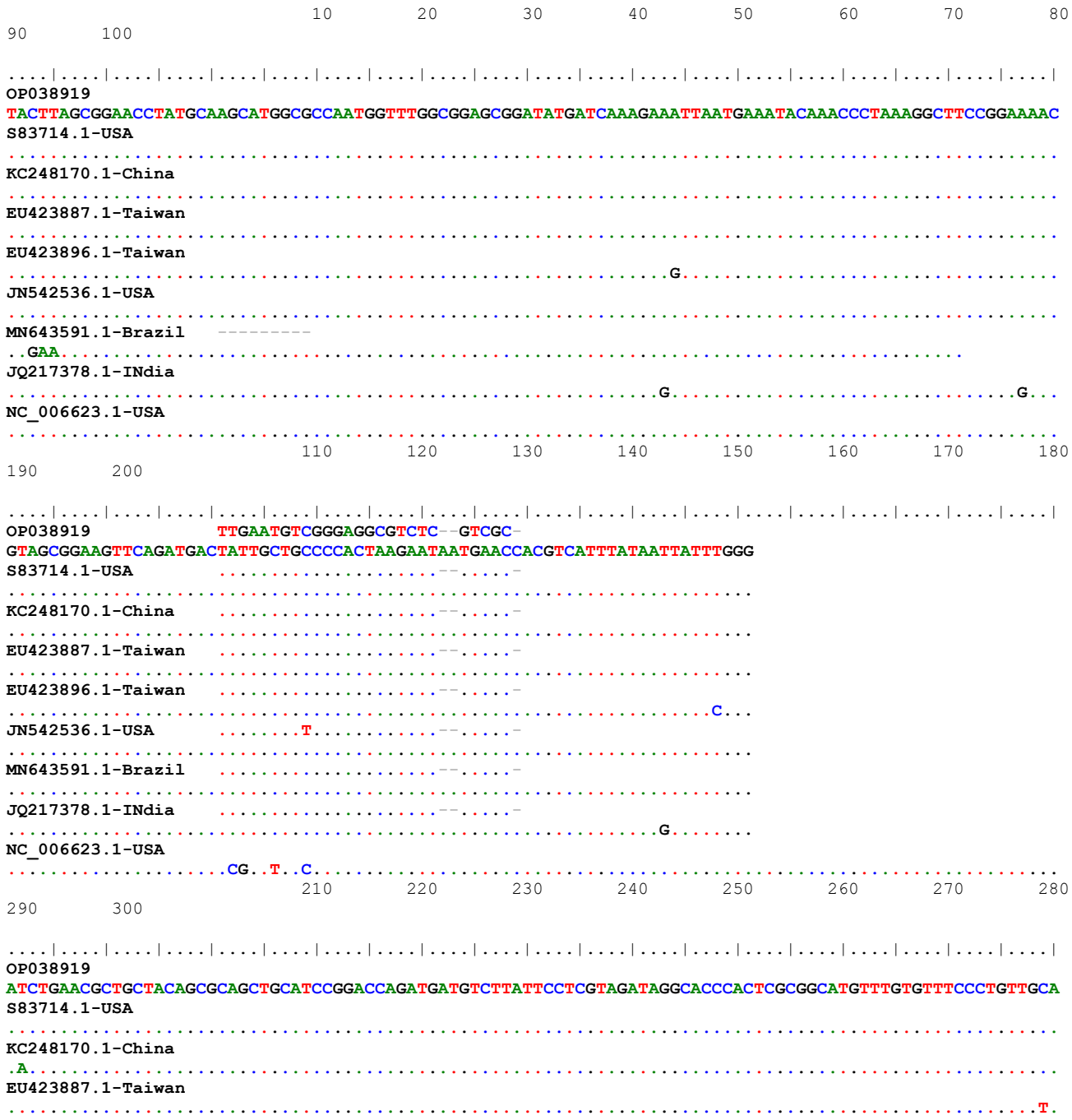
The DNA sequence of the isolates showed that there were several nucleotides substitutions within the sequence of the partially sequenced tdk gene (OP038919) in comparison to the sequence found in other taxons, such as S83714.1, KC248170.1, EU423887.1, EU423896.,

JN542536.1, JQ217378.1, and NC_006623.1. The DNA sequence differs at sites 193 and 194 (A with C) from MN643591.1, and at sites 243 and 244 (A with G) from JQ217378.1, EU423896.1. However, it differs at sites 277, 243, 289, 377, 382, and 372 from other taxons, respectively (Figures-1 and 2).

Some of those nucleotide substitutions led to amino acid chain alterations, such as the nucleotides substitution at the site 244 that altered the corresponding codon and caused the replacement of the amino acid Isoleucine (OP038919) with Valine if compared to taxon EU423896.1, and the nucleotide substitution at the site 382 led to a replacement of the amino acid Serine (OP038919) with Threonine if compared to KC248170.1. In addition, there were several other amino acid substitutions (Figure 3).

There was a significant association between infection, microscopic and molecular results, Chi-square (X^2) = 6.876; df = 1; P value = 0.008735431; ($P \leq 0.05$). Sequenced and BLAST results are recorded in "the National Center for Biotechnology Information (NCBI) Gene bank" as T equi in Baghdad racing horses, in identification numbers: ON641879.1 to ON641891.1.

Drawing The phylogenetic tree in (Figure 3) observed our isolates had highest similarity of 93.03-100% with 94-100% site coverage with Brazil, China, Jordan, India, Iran, Japan, Scotland, Morocco, United states of America, and Thailand isolates.



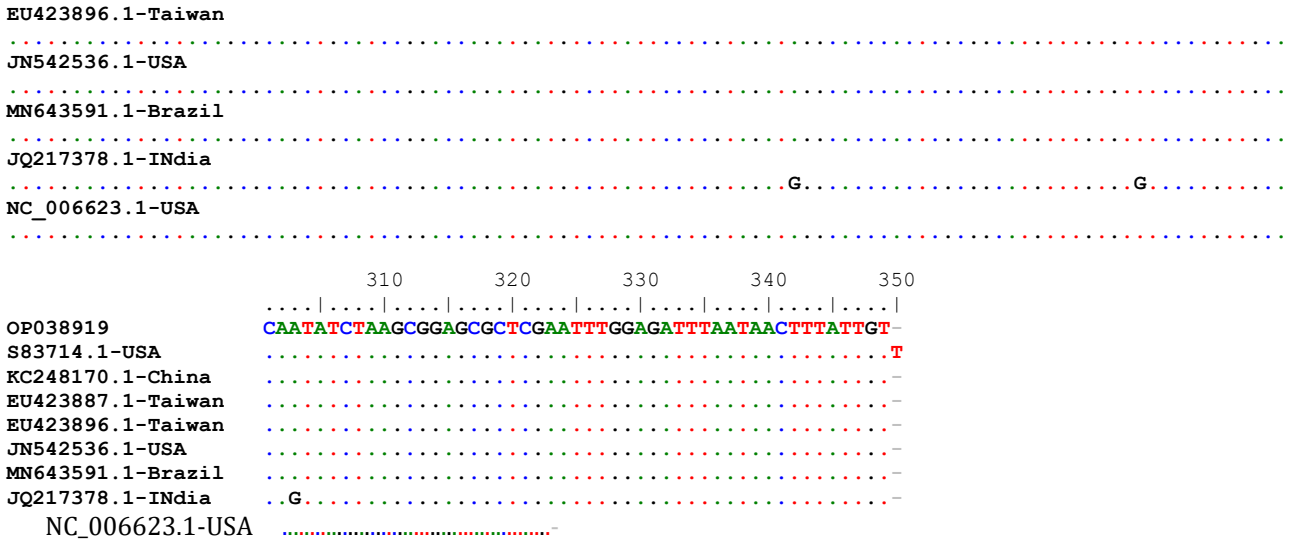


Figure 1. Partially sequenced tdk gene sequence of infectious laryngotracheitis virus alignment with other reported taxons

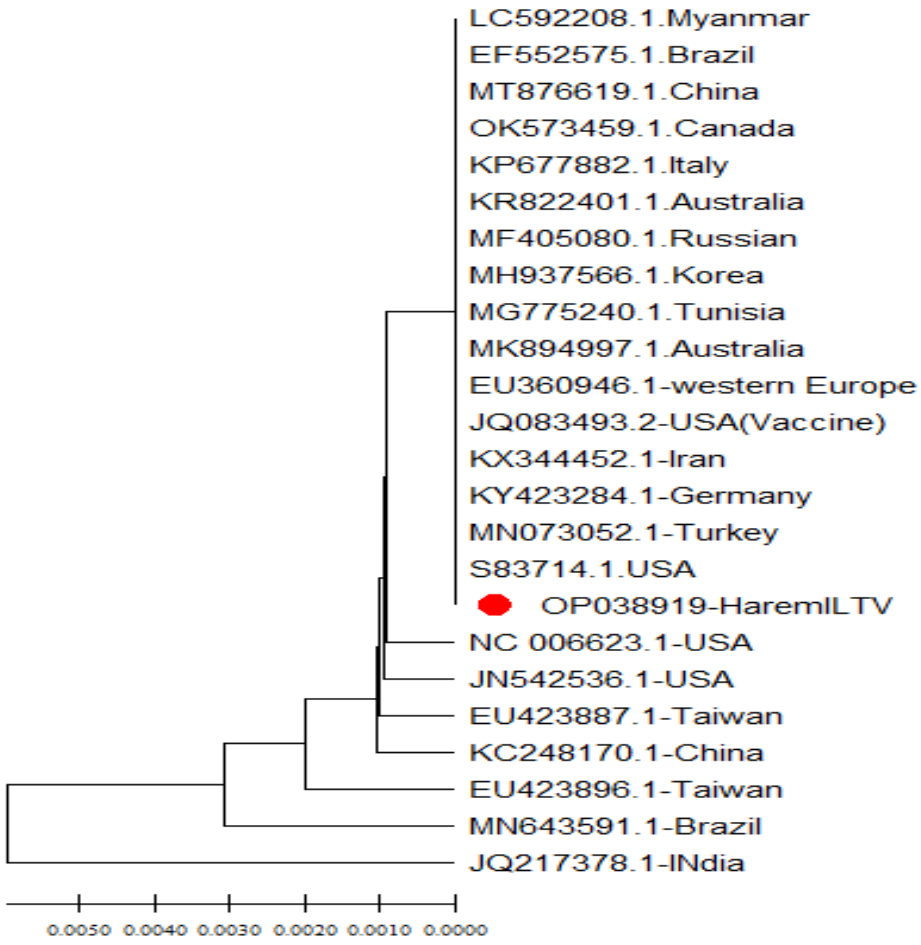


Figure 2. Phylogenic tree analysis of the partially sequenced tdk gene of infectious laryngotracheitis assigned by red spot

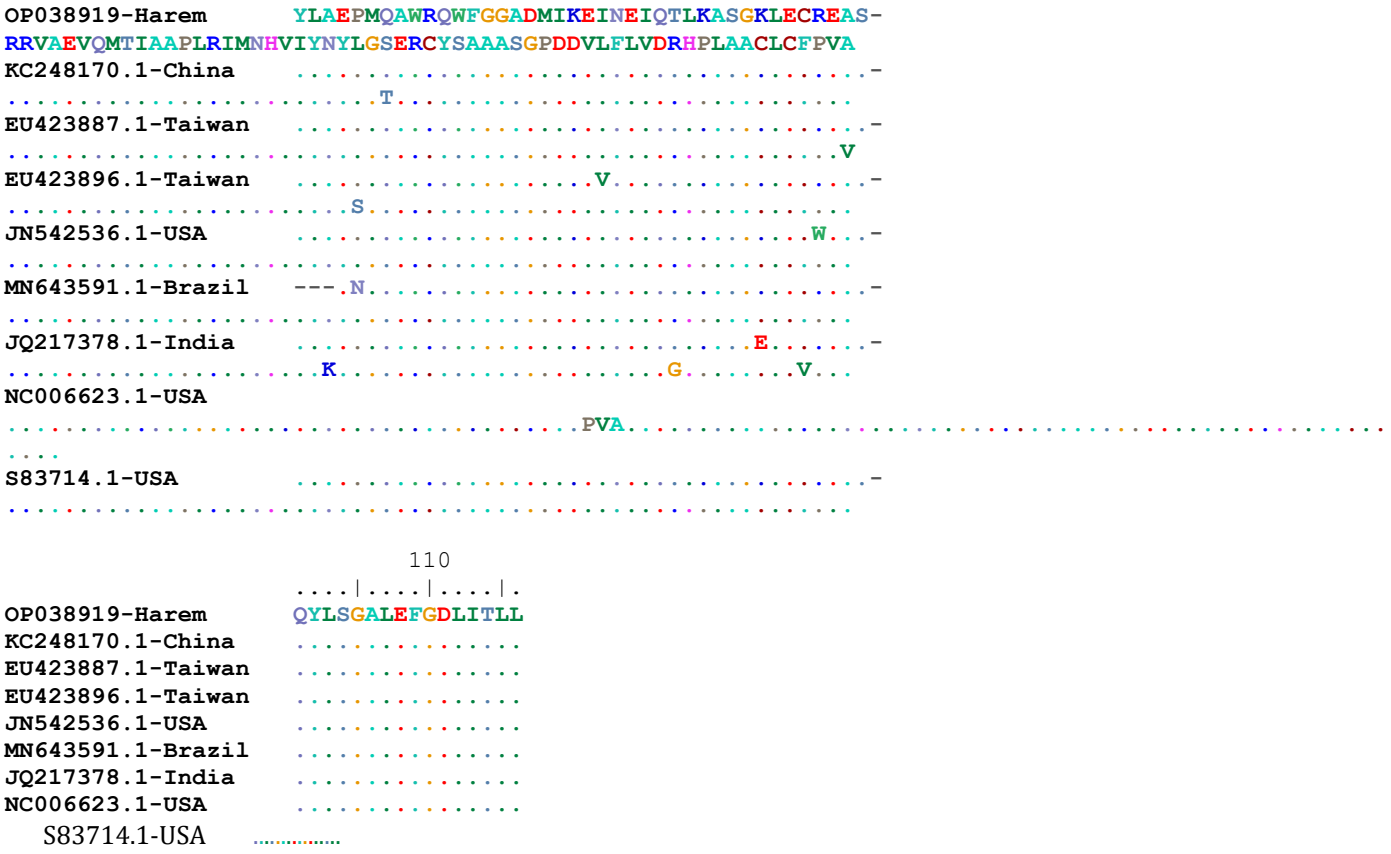


Figure 3. Amino acid sequence of the partially sequenced tdk gene aligned with other reported sequences

DISCUSSION

Despite its severity and economic importance, to the best of our knowledge, there was no any data in Sulaimaniyah province/Iraq about the incidence of ILTV infection in broiler farms. Therefore, this study investigated the incidence of the disease among chickens in that region, particularly among broilers that had clinical signs of respiratory diseases. During the study, 89 broiler farms in the region were investigated using polymerase chain reaction (PCR). The results showed that the frequency of the infection among broiler farms was about 2.2% (2/89). The infection was reported during January and February, and there were no associations between farm capacity, scaled-time schedule, vaccination of the checks with other vaccines and the rate of infection by ILTV. Similarly in Kermanshah province/Iran, the infection with ILTV was reported in 20-day old broiler chicks in February/2019 (6). A case report study in the southern united states showed a mild clinical manifestation of ILTV infection in 33-37 days old chicken, and the researchers could confirm the incidence of the disease even in two-weeks age chicks by inoculation, and they revealed an association between ILTV vaccination and the incidence of the disease (20). In Greece, ILTV infection was also detected in 28-day old organic broiler farms and the outbreak was linked to the ILTV vaccination in the region (21). Another study in the southern region of Myanmar, where the incidence of the disease in three different regions was investigated in laying hens, showed occurrence of ILTV infection during wet but not dry seasons (22).

The detected ILTV in the region had 100% similar to MN643591.1 and S83714.1, which were isolated from frozen trachea tissue of Gallus gallus in Brazil and chicken in the USA, respectively. DNA mutations, which had been recognized between current tdk-gene and other reported sequences in Gen/Bank indicated few alteration in amino acid sequences, that might alter the viruses behavior and pathogenesis (23, 24). The sequence analysis results indicated that the virus might originate from Brazil or the USA. Some of those microorganisms might had been introduced into the poultry-industry in Iraq, especially from Brazil and the USA, through imported poultry products (25), including frozen chicken meat (26)

This virus is more likely to infect almost all age groups. However, the clinical signs might be more obvious and severe in older ages (4, 12). The absence of typical ILT lesions and thier complication with other respiratory viral diseases, especially in the younger ages requires an efficient and supportive ancillary diagnostic test, such as PCR (27).

There was misleading information about the incidence of the disease and the manifestation of the clinical signs in young birds (28) that need to be further investigated. Increasing the biosecurity program and excluding life-attenuated vaccines of ILTV seems to be the most efficient way to control or limit the incidence of ILT caused by this virus. Whereas, using live–attenuated ILTV vaccine, particularly the chicken embryo origin (CEO) type, may revert their virulence efficiency (6, 29, 30).

The current study was the first that reported ILTV infection as one of the causes of respiratory infections in broiler farms in Sulaimaniyah province. The nucleotide sequencing of the viral tdk gene followed by phylogenetic tree analysis revealed that the virus isolate had a resemblance or difference with other worldwide reported serovars.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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تحري عدوى فيروس التهاب الحنجرة والرغامى المعدية في قطعان الدجاج اللاحم في محافظة السليمانية، العراق

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الخلاصة

التهاب الحنجرة والرغامى المعدية (ILT) هو عدوى حادة معدية في الجهاز التنفسي العلوي للدجاج والطيور الأخرى، يسببها فيروس Gallid herpesvirus1 (GaHV-1) ، الذي له أهمية اقتصادية في صناعة الدواجن. لاتوجد بيانات علمية حول نسبة الإصابة بالمرض في قطعان الدجاج اللاحم في محافظة السليمانية / العراق. لذلك هدفت هذه الدراسة إلى تحرى الاصابة بمرض ILTV في قطعان الدجاج اللاحم في المنطقة. تم فحص الطيور المصابة سريريا من ٨٩ من قطعان الدجاج اللاحم التي تعاني من ضيق في التنفس والسعال واللهاث وقشور القصبة الهوائية وإفرازات الأنف العينية واحتقان القصبة الهوائية مع إفراز صديدي والتهاب القصبات النزفية مع / أو بدون تغيرات نخرية. تم استخراج الحمض النووي من العينات المجمعة ، بما في ذلك إفرازات الرغامى والرغامى وأنسجة الرئة. جرى استخدام البواديء الخاصة بجين thymidine kinase (tdk) من ILTV- في تفاعل البوليميراز المتسلسل للكشف عن الفيروس. تم إنشاء شجرة فيلوجينية لتتبع أصل الفيروس. وكشفت الدراسة أن نسبة الإصابة ب ILTV بين قطعان الدجاج اللاحم كان ٢،٢٪ (٨٩/٢) في المنطقة. أظهر تحليل التسلسل أن ILTV المعزول في المنطقة كان مرتبطا ارتباطا وثيقا بالسلالات المبلغ عنها في الولايات المتحدة والبرازيل (MN643591.1 و S83714.1) ، وكان له هوية تسلسلية تبلغ ٩٨،٢٧٪ إلى التصنيف JQ217378.1. في الختام ، افادت الدراسة أن أحد أسباب العدوى الفيروسية التنفسية في قطعان التسمين حتى في الأعمار الأصغر كان مرتبطا ب ILTV. أظهر جين tdk المتسلسل جزئيا للفيروس أن serovars المنتشر في المنطقة يحتوي على بعض الاختلافات في النيوكليوتيدات والأحماض الأمينية مع serovars في جميع أنحاء العالم. يجب أن يؤخذ هذا في الاعتبار في صناعة الدواجن من خلال إجراء مزيد من التحقيقات.

الكلمات المفتاحية: التهاب الحنجرة والرغامي المعدي، الدجاج اللاحم، السليمانية