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A surveillance for avian coronavirus infectious bronchitis virus, infectious laryngotracheitis virus, avian metapneumovirus, and avian reovirus in poultry flocks with respiratory signs in Türkiye

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Abstract: In this study, avian coronavirus infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), avian metapneumovirus (AMPV), and avian reovirus (ARV) were evaluated in broiler and layer flocks. For this purpose, tracheal swabs from 48 broiler and 45 layer flocks with respiratory signs were inoculated SPF embryonated chicken eggs for virus isolation. The viruses were identified by real-time PCR. Results showed that the most common virus in both broiler and layer farms was IBV with incidence rates of 58.33% and 46.67%, respectively. ILTV, AMPV, and ARV incidences in the samples were found to be 22.22%, 13.33%, and 4.44% in layer flocks while 2.08%, 8.33%, and 20.83% in broilers, respectively. The numbers of IBV+AMPV and IBV+ARV coinfections were 5 (11.11%) and 1 (2.22%) in layers, whereas, 1 (2.08%) and 5 (10.42%) broilers, respectively. In addition, 2 broiler flocks (4.17%) had triple infection with IBV, AMPV, and ARV. ILTV was detected always alone from the samples of layer and broiler flocks. Sequencing of *S1* gene of selected IBV TR/L45 and TR/B42 isolates showed similarities with IS/1494/06 (HM131453) at the rates of 98.98% and 99.69%, respectively, while TR/L37, TR/L38, and TR/L39 isolates were identical to 4/91 (KF377577) vaccine strain at the rates of 99.01%, 99.01%, and 98.76%, respectively. Sequencing analysis of the *ICP4* and *TK* genes of ILTV isolates revealed that they were all field strains with low virulence. The present data represent actual information on the genotypes of IBV and ILTV circulating in poultry flocks in Türkiye.

Key words: Infectious bronchitis virus, infectious laryngotracheitis virus, avian metapneumovirus, avian reovirus, qPCR, chicken

1. Introduction

Viral respiratory tract infections lead to important economic problems for the poultry industry worldwide. They exhibit variable mortality rates and severe clinical symptoms such as respiratory tract disease, reduced egg, and meat yield, and decreased egg quality [1]. These similar clinical and pathological pictures lead to serious difficulties in clinical differential diagnosis, particularly in cases of mixed infection [2]. Therefore, screening of respiratory tract viruses including avian coronavirus infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), avian metapneumovirus (AMPV), and avian reovirus (ARV) is important for accurate diagnosis and protection [2-5].

IBV, a member of the family *Coronaviridae* is a single-stranded, and positive-sense RNA virus encoding several proteins associated with RNA replication and transcription. Among these proteins, spike (S) protein, which cleaved into S1 and S2 subunits, mediates host cell attachment and fusion of the viral and cellular membranes. The *S1* gene coding for the S1 protein has a hypervariable region that is liable to mutation or recombination. Thus, genetic variability caused by nucleotide insertions and deletions at different locations in the spike gene leads to emerging of novel IBV variants. Therefore, analysis of *S1* using reverse transcription-polymerase chain reaction (RT-PCR) and sequencing is crucial to identify IBV genotypes [6]. In this manner, the epidemiology and evolution of the IBV can be better understood and appropriate vaccines can be developed or selected to achieve efficient control of the disease. Numerous different IBV genotypes have been detected worldwide. Moreover, new variants continue to emerge in different parts of the world [7]. In Türkiye, the first isolation of IBV was reported by Kahya et al. [8]. Their results showed that IBV isolates were related to IS/1494/06. In subsequent studies, Ma5, H120, and 4/91 genotypes were also detected [9, 10].

ILTV is referred to as *Gallid herpesvirus 1* (GaHV-1) belonging to the family *Herpesviridae*. The genome of ILTV, which is linear double-stranded DNA, is composed

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of unique long (U_L) and unique short (U_S) regions, and two inverted repeat regions flanking the U_S region. Currently, sequencing analysis of the infected-cell protein 4 (*ICP4*), located in inverted repeats, enables effective detection of the differentiation between field and vaccine isolates [11]. Thymidine kinase (*TK*) gene, located in the UL region, determines the virulence of the virus. The presence of a deletion in the 272 to 283 bp region of the *ICP4* gene can be used as an indicator for the vaccine strains of the virus. On the other hand, the absence of the T252M mutation has been reported to be a characteristic genomic alteration for strains with low virulence. To control the ILT, live attenuated vaccines are used. Whole-genome sequence analyses of vaccine and field strains indicate that live attenuated vaccine viruses can revert to virulence after bird to bird passage [12]. Additionally, it is worth noting that vaccine strains can cause the disease after establishing latent infections [13]. In Türkiye, some studies have shown the presence of ILTV from layer and broiler flocks [14, 15]. It is important to note that molecular epidemiological studies are needed to compare the ILTV strains involved in severe and mild outbreaks and to characterize the circulating virus in poultry flocks.

Recent studies have indicated growing concern about the role of AMPV in avian respiratory outbreaks worldwide. This virus belongs to the genus *Metapneumovirus* of the family *Paramyxoviridae* and it causes issues of the upper respiratory tract and decreases in production parameters such as egg yield and shell quality. Although the presence of AMPV was previously reported in Türkiye [16], there is limited information on the prevalence of AMPV and its concomitance with other respiratory viruses in broiler and layer flocks.

ARV is a member of the genus *Orthoreovirus* of the family *Reoviridae*. This virus is highly associated with many diseases including arthritis, myocarditis, hepatitis, tenosynovitis, and enteric/respiratory diseases and syndromes such as malabsorption and stunting in poultry [17]. Thus, ARV infections cause strikingly decreased profits for the poultry industry. After the first serological detection of ARV by Carli et al. [18], the virus was observed by different researchers in various regions of Türkiye. However, the molecular data on its distribution and the current status in broiler and layer flocks is rather limited.

As in the whole world, avian respiratory viruses produce significant economic losses in poultry flocks due to mortality, weight loss, reduced egg production, and susceptibility to seconder infections in the national poultry industry. However, the number of studies based on molecular aspects is quite limited in Türkiye; hence, there is still a strong need for analyses regarding ways to detect agents and to determine their subtypes. To the best of our knowledge, this is the first report to collectively determine the frequency of IBV, ILTV, AMPV, and ARV infections in Türkiye.

The objective of this study was to investigate the current field situation of respiratory tract viruses including IBV, ILTV, AMPV, and ARV in broiler and layer flocks by using a quantitative real-time-polymerase chain reaction (qPCR) in commercial poultry flocks located in different regions of Türkiye. Furthermore, IBV and ILTV were molecularly identified to monitor the genetic features of the circulating viruses.

2. Materials and methods

2.1. Sampling

Tracheal swabs were collected from 48 broiler and 45 layer flocks with respiratory problems in 15 different provinces of Türkiye (İzmir, Aydın, Uşak, Denizli, Manisa, Afyon, Balıkesir, Bursa, Sakarya, Bolu, Ankara, Kayseri, Adana, Elazığ, Van). Sampling was done in almost all geographical areas of Türkiye. Samples were obtained from avian influenza and Newcastle disease-free flocks confirmed by the virus isolation in specific pathogen-free (SPF) embryonated chicken eggs (ECE) and RT-PCR. These flocks showed respiratory problems such as tracheal rales, gasping, sneezing, coughing, and sinusitis. Initially, five samples per flock were obtained from the birds showing respiratory symptoms. Afterward, these 4–5 samples were pooled and regarded as one sample for each corresponding flock. Eventually, a total of 93 samples were placed in tryptose phosphate broth (Becton Dickinson, 260300) containing penicillin (10,000 International Units [IU]/mL), streptomycin (10 mg/mL), and amphotericin B (25µL/mL) (Sigma-Aldrich, A5955). Tracheal swab suspensions were centrifuged at 3000 rpm for 10 min and filtrated through bacteriological filters (Sartorius Stedim Biotech).

2.2. Virus isolation

Virus isolation was performed in SPF ECE. For IBV isolation, 200 µL aliquots of the suspensions were inoculated into the allantoic cavity of 9–11-day-old ECEs[1], whereas 100 µL of samples were inoculated into the chorioallantoic membrane (CAM) of 10–12-day-old ECEs for ILTV[2]. In addition, 9–11-day-old ECEs were used for ARV isolation by means of inoculation of 100 µL of samples to CAM [19]. Regarding AMPV, inoculation of 200 µL sample to yolk sac of 6–8-day-old ECEs was performed

[1] World Organisation for Animal Health (2018). OIE Terrestrial Animals, Avian Infectious Bronchitis [online]. Website https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.02_AIB.pdf 2018 (accessed 13 March 2022).

[2] World Organisation for Animal Health (2021). OIE Terrestrial Animals, Avian Infectious Laryngotracheitis [online]. Website https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.03_AVIAN_INF_LARYNGO.pdf 2021 (accessed 13 March 2022).

to provide adequate isolation of the virus[3]. Samples were blindly propagated by at least three passages according to the standard procedures. The eggs were incubated at a temperature of 37 °C for 4–7 days. Eggs were candled daily for 7 days and, in this period, mortality within the first 24 h was considered nonspecific. Concerning nucleic acid extraction, a total of 2 mL samples were obtained from allantoic liquid, CAM, and yolk sac material.

2.3. Nucleic acid extraction

Nucleic acids were extracted using QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amount and purity of the DNA or RNA samples were determined using spectrophotometry (NanoDrop 2000c, Thermo Scientific, Wilmington, DE, USA). The samples were stored at –20 °C until analysis.

2.4. qPCR analysis for virus detection

The primers were selected from the published data for the amplification of IBV, ILTV, AMPV, and ARV, as shown in Table 1. To detect the IBV, a real-time Taqman PCR assay was used as described by Callison et al. [20]. Detection of ILTV was performed by using QuantiTect SYBR Green RT-PCR Kit (Qiagen) (without reverse transcriptase). Amplifications for the detection of AMPV and ARV were conducted with QuantiTect SYBR Green RT-PCR Kit (Qiagen).

2.5. Presequencing PCR procedures

A nested PCR assay was performed using a thermal cycler (BioRad C100 Touch Thermal Cycler, BioRad Laboratories, California, USA) to amplify the *S1* gene of IBV. Initial PCR reaction was carried out in a mixture containing 14.5 µL of ddH_2O, 0.5 µL of the primers SX1 and SX2, 2.5 µL of RNA, 1 µL of OneStep RT-PCR Enzyme Mix (Qiagen), 5 µL OneStep RT-PCR Buffer (Qiagen), and 1 µL dNTP mix (Qiagen). Nested PCR was performed in a mixture of 20 µL containing 7.2 µL of ddH_2O, 0.4 µL of the primers SX3 and SX4, 2 µL of the first amplicon, and 10 µL of Taq PCR master mix (Qiagen). Concerning ILTV, amplification of the *ICP4* and *TK* genes was performed in a total volume of 25 µL containing 6.5 µL of ddH_2O, 12.5 µL of 2x QuantiTect Probe PCR master mix, 0.5 µL of each primer, and 5 µL of the DNA. Corresponding primers were selected from the relevant studies for IBV and ILTV, as shown in Table 2. Amplification products were determined by agarose gel electrophoresis (1.5%) and visualized with a gel imaging system (Gel Doc 2000, BioRad Laboratories, USA) after staining with SYBR Green I.

2.6. Sequence and phylogenetic analysis

Five selected isolates of IBVs were genotyped by partially sequencing of *S1* region. In addition to IBV, five selected ILTV isolates were genotyped and evaluated by phylogenetic analyses. Amplification products were purified and were sequenced using an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer). Multiple sequence alignments were performed using Jalview v2.7. Afterward, aligned sequences were subjected to bootstrap (1000 replicates) and a phylogenetic tree was constructed with MEGA 7 v7.0.26 by the neighbor-joining method with Kimura two-parameter model.

2.7. Accession numbers

The sequences in this study were deposited in the GenBank database as follows: for *S1* gene, L45, OK665844; L38, OK665845; L39, OK665846; B42, OL956526; L37, OL956527, for *ICP4* gene L8, OL956532; L40, OL956533; L23, OL956534; L35, OL956535, for *TK* gene L8, OL956528; L41, OL956529; L23, OL956530; L35, OL956531. Accession numbers of IBV and ILTV which are used in the phylogenetic analysis are presented in Table S1.

3. Results

3.1. Epidemiological data

The number and percentages of the observed positive samples and their distributions concerning flock types are presented in Table 3. In this context, the highest percentage of infection in layer and broiler flocks was detected in IBV (46.67% and 58.33 %, respectively). Positive samples were originated from ten different provinces (Manisa, İzmir, Uşak, Afyon, Sakarya, Balıkesir, Kayseri, Elazığ, Adana, Van). ILTV which was detected at the lowest rate in broiler flocks (2.08%) was the second-highest infection in layer flocks (22.22%). Regarding the total number of positive samples, ILTV, AMPV, and ARV have relatively similar frequencies (11.83%, 10.75%, and 12.90%, respectively) compared to IBV infections. The samples of the 12 layer and 15 broiler flocks were all negative for the corresponding viruses. All birds were negative for AIV and NDV; hence, they will not be discussed further.

Concerning the evaluation of multiple infections, results revealed that ILTV did not show a combined infection with any of the studied viruses. IBV and AMPV coinfection was detected in one (2.08%) of 48 broiler samples while it was seen in 5 (11.11%) out of 45 layer samples. On the contrary, IBV and ARV coinfection was detected in 5 broilers (10.42%) and 1 layer (2.22%) samples. Moreover, triple infection was only seen in broiler flocks with a low frequency (4.17%). In this respect, only two flocks had IBV, AMPV, and ARV coinfection.

3.2. Genomic alterations

Compared to *S1* gene sequences of IS/1494/06 (HM131453) IBV, TR/L45 had one amino acid alteration at position

[3] World Organisation for Animal Health (2018). OIE Terrestrial Animals, Turkey Rhinotracheitis (Avian Metapneumovirus Infections) [online]. Website https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.15_TURKEY_RHINO.pdf 2018 (accessed 13 March 2022).

Table 1. Primers used for IBV, ILTV, AMPV, and ARV qPCR test.

Respiratory pathogen	Primer	Sequence(5′-3′)	PCR conditions	References
IBV	IBV5'GU391	GCTTTTGAGCCTAGCGTT	50°C 30', 95°C 15' (94°C 20 s, 60°C 1') 50 cycles	Callison et al. [20]
	IBV5'GL533	GCCATGTTGTCACTGTCTATTG		
	IBV5'G prob	FAM-CACCACCAGAACCTGTCACCTC-BHQ1		
ILTV	ILTp32 U2	CTACGTGCTGGGCTCTAATCC	95°C 15' (95°C 30 s, 55°C 40 s, 72°C 40 s) 50 cycles	Vögtlin et al. [35]
	ILTp32 L2	AAACTCTCGGGTGGCTACTGC		
AMPV	Nd	AGCAGGATGGAGAGCCTCTTTG	50°C 30', 95°C 15' (95°C 15 s, 56°C 45 s) 50 cycles	Bäyon-Auboyer et al. [36]
	Nx	CATGGCCCAACATTATGTT		
ARV	σA1	ATTACGCAGAGGCATTT	50°C 30', 95°C 15' (95°C 15 s, 56°C 45 s) 50 cycles	Ke et al. [37]
	σA2	CCCACTGCCGAATACA		

IBV: Avian coronavirus infectious bronchitis virus; ILTV: infectious laryngotracheitis virus; AMPV: avian metapneumovirus; ARV: avian reovirus; qPCR: quantitative real-time-polymerase chain reaction.

Table 2. Primers used for sequencing the *S1* gene of IBV and *ICP4* and *TK* genes of ILTV.

Pathogen	Gene	Primer	Sequence(5′-3′)	Amplicon size (bp)	Reference
IBV	*S1*	SX1+	CACCTAGAGGTTTGT/CTA/TGCAT	393 bp	Worthington et al. [21]
		SX2-	TCCACCTCTATAAACACCC/TTT		
		SX3+	TAATACTGGC/TAATTTTTCAGA		
		SX4-	AATACAGATTGCTTACAACCACC		
ILTV	*ICP4*(fragment 1)	F	GGGTCTTGTTCTGCAGGATTCT	923 bp	de Macedo Couto et al. [12]
		R	CATCGGGACATTCTCCAGGTAGCA		
	ICP4(fragment 2)	F	CTTCAGACTCCAGCTCATCTG	747 bp	
		R	AATGAGCACGCAACCAGAAGTAA		
	TK	F	CTTAGCGGAACCTATGCAAG	783 bp	
		R	GAGGCCATGTGCTGGTAAGT		

S1: Spike 1; IBV: avian coronavirus infectious bronchitis virus; *ICP4*: infected-cell protein 4; *TK*: thymidine kinase; ILTV: infectious laryngotracheitis virus.

365 (serine to asparagine), 387 (isoleucine to arginine), and 389 (glutamate to glycine). TR/L37, TR/L38, and TR/L39 had three amino acid alterations at position 365 (phenylalanine to tyrosine), 366 (serine to asparagine), and 390 (glutamate to glycine) compared to 4/91 (AF093794) IBV *S1* gene sequences. Besides, TR/L38 had one further amino acid alteration at position 391 (leucine to lysine) which causes a missense mutation. Most of the amino acid changes were located within the hypervariable region 3 (amino acid position 274–387) of S1 protein in this study.

Concerning mutations and their corresponding genomic positions, based on the *ICP4* gene (fragment 1) of the ILTV, two mutations were observed at position 735 (cytosine to thymine) and 814 (guanine to adenine) in TR/L40. According to the evaluation of the amino acid sequence, mutation at position 814 is a missense mutation because it leads to an amino acid alteration at position 272, recognizable as glutamate to glycine whereas mutation at position 735 is a silent mutation. No deletions were detected within the 272–283 bp regions in the sequences of *ICP4* fragment as observed in the sequences of CEO vaccine strains. In addition, no mutation was present in fragment 2. Concerning the *TK* gene, one mutation was observed at position 661 (adenine to thymine) in TR/L8. This nucleotide alteration led to asparagine to tyrosine change at position 221. There was no mutation at the amino acid position 252 in the *TK* gene; hence, the strain was considered low-virulent.

Table 3. Frequencies of IBV, ILTV, AMPV, and ARV in broiler and layer flocks in Türkiye.

	Broiler (n = 48)		Layer (n = 45)		Total (n = 93)	
	Number	Percentage	Number	Percentage	Number	Percentage
IBV	28	58.33%	21	46.67%	49	52.69%
ILTV	1	2.08%	10	22.22%	11	11.83%
AMPV	4	8.33%	6	13.33%	10	10.75%
ARV	10	20.83%	2	4.44%	12	12.90%
Negative samples	15	31.25%	12	26.67%	27	29.03%

IBV: avian coronavirus infectious bronchitis virus; ILTV: infectious laryngotracheitis virus; AMPV: avian metapneumovirus; ARV: avian reovirus.

3.3. Phylogenetic analysis

In this study, phylogenetic analysis of the *S1* gene of five IBV isolates assigned the strains into two different clusters. TR/L45 and TR/B42 were clustered into the genotype I (GI)-23 lineage while TR/L37, TR/L38, and TR/L39 were clustered into GI-13 lineage (Figure 1). BLASTN analysis showed that the *S1* gene of TR/L45 and TR/B42 shares 98.98% and 99.69% similarity with IS/1494/06 (HM131453). Moreover, TR/L37, TR/L38, and TR/L39 were closely related (99.01%, 99.01%, and 98.76%, respectively) to the 4/91 (KF377577) vaccine strain.

Phylogenetic analysis of the partial *ICP4* gene showed that our four ILTV strains (TR/L8, TR/L40, TR/L23, TR/L35) were clustered with the CEO vaccine (EU104900) strain with the similarity of 97.74% to 98.52%. The sequences obtained in this study are located in the same cluster with previously reported Turkish isolates (Figure 2). Concerning the phylogenetic evaluation based on the *TK* gene, Turkish isolates including TR/L23, TR/L35, TR/L41, and TR/L8 were clustered in the same lineage with previously detected Turkish isolates (Figure 3).

4. Discussion

Evaluation of adequate control strategies in respiratory diseases should definitely be performed to decrease economic losses caused by high mortality rates and decline in yields. Despite routine vaccination against respiratory diseases, outbreaks of these diseases frequently occur in the field because of the presence of different strains and novel variants generated by genomic alterations including point mutations and recombinations. In this respect, advances in molecular diagnostic techniques have enabled researchers to evaluate comprehensive data in discerning the mechanisms responsible for respiratory diseases because these techniques can provide highly specific biological information at the molecular level. In the present study, the main objective was to determine the current status of respiratory infections including IBV, ILTV, AMPV, and ARV, and thus, to monitor the continuous emergence of novel genetic variants in Türkiye.

In this study, IBV was the most common (52.69%) in the samples from chickens with respiratory signs. This indicated that IBV is still an important problem in Turkish poultry flocks despite the immunization of numerous poultry flocks with inactivated and live attenuated vaccines. Outbreaks of IBV infections occurred due to prevalent mutations in the *S1* gene. These genomic alterations may result in the formation of different serotypes and genotypes in the field [21]. In the present study, the prevalence rate of IBV was 52.69% and the corresponding genotypes were IS/1494/06 (HM131453) and 4/91 (KF377577). In this context, 4/91 (KF377577) was more frequent compared to the other detected type, IS/1494/06 (HM131453). The phylogenetic analysis of the complete genome was conducted to investigate the relationship of the obtained IBV variants with different virus genotypes previously submitted to GenBank (16 IBVs were selected which represent relevant territories and lineages). Regarding layer flocks, three IBV strains were closely related to 4/91 (KF377577) while one of them showed a high genomic similarity to IS/1494/06 (HM131453). In accordance with the present results, Yilmaz et al. [10] indicated that in layer flocks all sequences were 4/91 vaccine strain (96.2%–99.5%) in Türkiye. Recent studies on IBV prevalence have shown that the 4/91 vaccine strain is the most frequently detected IBV type in different regions of the world. In this respect, de Wit et al. [22] reported that strains of the 4/91 were the predominant genotype in several European countries including Germany, France, Italy, the Netherlands, Greece, Poland, Portugal, the Republic of Ireland, Spain, and the UK. Furthermore, RT-PCR analyses by Ganapathy et al. [23] have also revealed that remarkably higher frequency (43.66%) was observed for the 4/91 genotype compared to other detected genotypes. The prominence of this genotype is not surprising because 4/91 vaccine strains are widely used in the Middle East. In

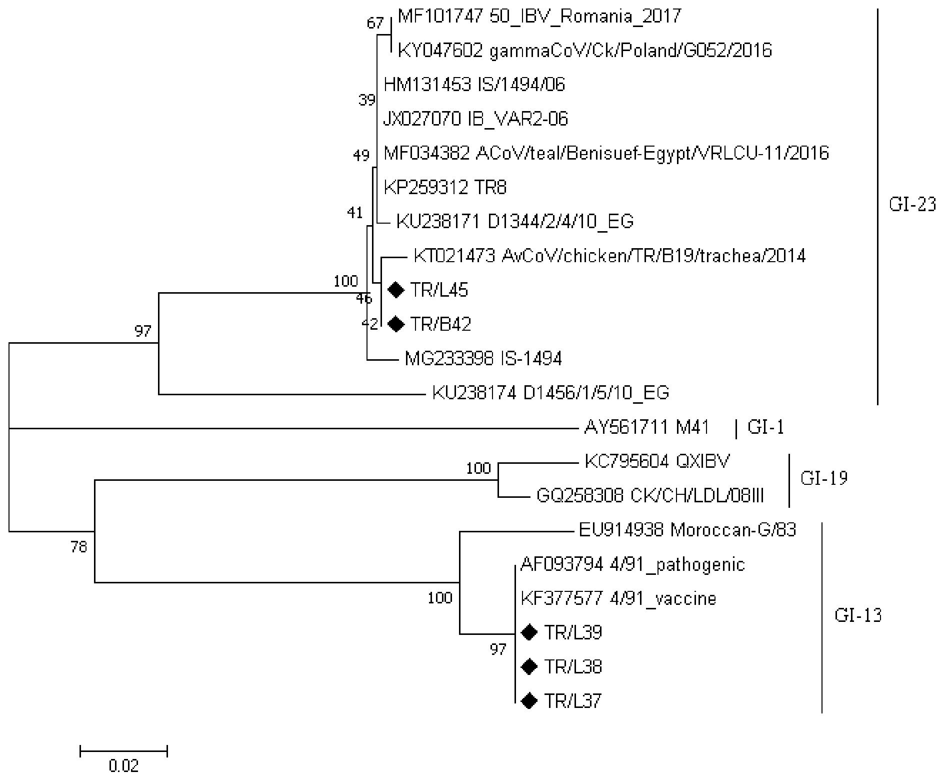

Figure 1. Phylogenetic tree regarding a partial sequence of the spike 1 (*S1)* gene, which indicates the relationship between the strains in Türkiye and previously described avian coronavirus infectious bronchitis virus (IBV) strains. This tree was generated using the neighbor-joining method with the Kimura 2-parameter substitution model and 1000 bootstrap replications. Türkiye isolates in the current study are indicated by black diamonds. The scale bar demonstrates the distance unit between sequence pairs. The sequences were acquired from GenBank.

this study, the phylogenetic comparison of the partial S1 sequences revealed that sequences from the broiler flock were closely related to IS/1494/06 (99.69%). Supportively, Kahya et al. [8] reported that IBV strains isolated from tracheal swab samples of breeder chicken and broiler flocks were related to IS/1494/06 with 99 % identity. Moreover, Yilmaz et al. [10] suggested that the most common type of IBV in Türkiye belongs to Israel variant 2 isolates with 94.8%–99.4% similarity. The results obtained from the present study and the previously published reports in Türkiye support the idea that the predominance of IBV genotypes was transferred from Middle East countries because of widely intensive poultry trade with Middle Eastern countries compared to European countries. To prevent IBV outbreaks in the Turkish poultry industry, chickens are routinely vaccinated with multivalent IBV vaccines which contain heterogeneous genotypes including 4/91 and IS/1494/06. However, it is important to note that, using appropriate protectotype vaccines against IBV is essential to provide sustainable immunity in the field. On the other hand, the routine application of IBV vaccines in the field may involve many variations in application techniques and their components including quality, quantity (adequate intervals and the optimized doses), and the combination of different vaccines. Further studies are needed to perform more reliable and applicable analyses on vaccination programs. Moreover, the evaluation of genotypic characteristics of vaccine, field, and the reverting vaccine strains should be carried out to develop effective protectotype vaccines.

ILTV is a herpes virus that replicates in respiratory mucosa and it continues to cause serious outbreaks of respiratory disease in chickens and other bird species worldwide. In this study, the frequency of occurrence rate was 11.83% in layer and broiler flocks. Sequencing of the *ICP4* gene, which is a specific genomic region of ILTV, can provide reliable clues for differentiation between field and vaccine strains. According to the sequencing analysis of *ICP4*, there were no deletions within the 272–283-bp region of this gene. The presence of a deletion in this genomic location has been reported to be a specific characteristic for CEO and TCO vaccine strains [12, 24]. Thus, the sequences obtained in the present study indicated that the chickens were infected by a field virus.

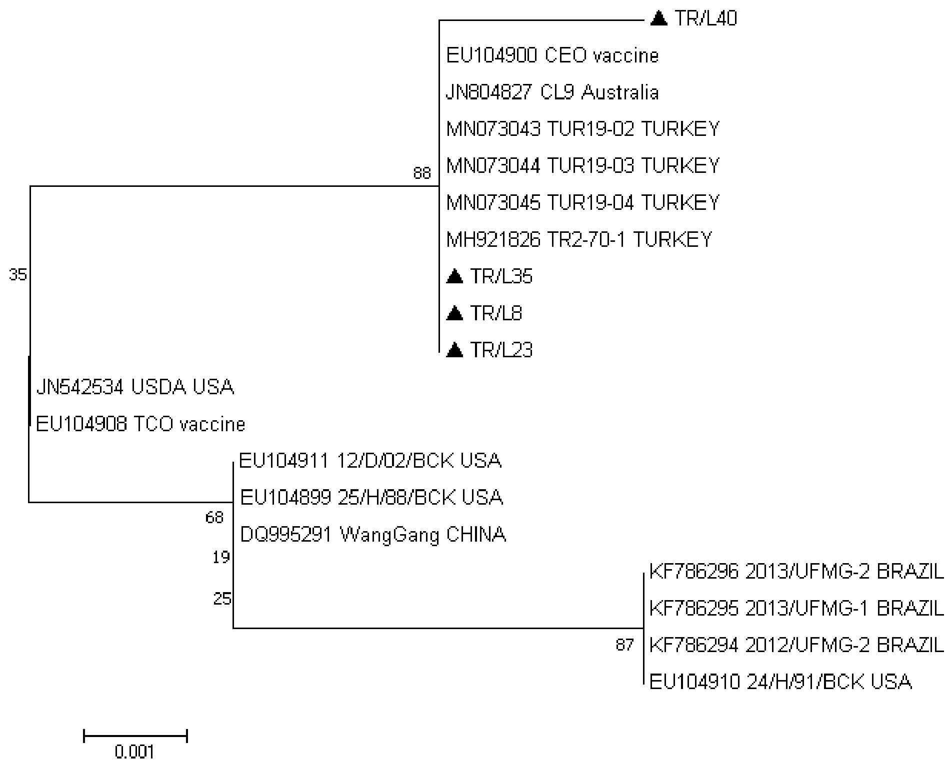

Figure 2. Phylogenetic tree regarding a partial sequence of the infected-cell protein 4 (*ICP4*) gene, which indicates the relationship between the Türkiye strains and other infectious laryngotracheitis virus (ILTV) strains. This tree was generated using the neighbor-joining method with the Kimura 2-parameter substitution model and 1000 bootstrap replications. Türkiye isolates in the current study are indicated by black triangle. The scale bar demonstrates the distance unit between sequence pairs. The sequences were acquired from GenBank.

Another important genomic region of ILTV is the location of the *TK* gene because a fragment of this sequence, at least partially, can be used to differentiate isolates of high or low virulence. In this sense, low virulence strains specifically possess a threonine at position 252 of the *TK* gene, while methionine, at the same position, is a characteristic property of strains with high virulence [12, 25]. In the present study, the sequences indicate that there was no threonine/methionine alteration at the corresponding genomic region; hence, the strains might be low-virulent. It is important to note that very few reports are available on the existence of the ILTV in the Turkish poultry population. The first report about ILT disease in broiler breeders in Türkiye was published by Kaya and Akan [15] and it suggests that samples share 99% sequence identity with wild-type strains from different countries and also with commonly used live vaccines. Moreover, Can-Sahna et al. [14] suggested that the nucleotide sequences of the *TK* and *ICP4* gene regions of the ILTV isolates share more than 95% identity with other isolates from different various countries, such as the USA, Russia, and China. In accordance with our results, these researchers reported that the strains were low-virulent. Although the present study and the results from previously published reports indicate that the circulating virus is a low-virulent wild-type in the Turkish poultry field; further whole-genome studies of ILTV are necessary to advance the understanding of the molecular genetic characteristics of the Turkish strains. Some other important genomic regions, for instance, the UL47, gG, gE, ICP18.5, and ORFB-TK regions can be included to support these findings and to evaluate other possibilities [26, 27]. Many researchers have suggested that vaccine strains gradually replace the wild-type viruses in the field and their virulence increases due to the process of flock-to-flock transmission [28]. On the other hand, spontaneous recombination of various strains can lead to the emergence of novel virulent strains in the field. Taken altogether, comprehensive sequencing studies of the different strains should be critically conducted to evaluate the current vaccination strategies and biosecurity applications and to prevent future ILT outbreaks.

To prevent and control ILTV outbreaks, modified live virus vaccines have been developed and released in many countries. Despite their efficacy, live vaccines can

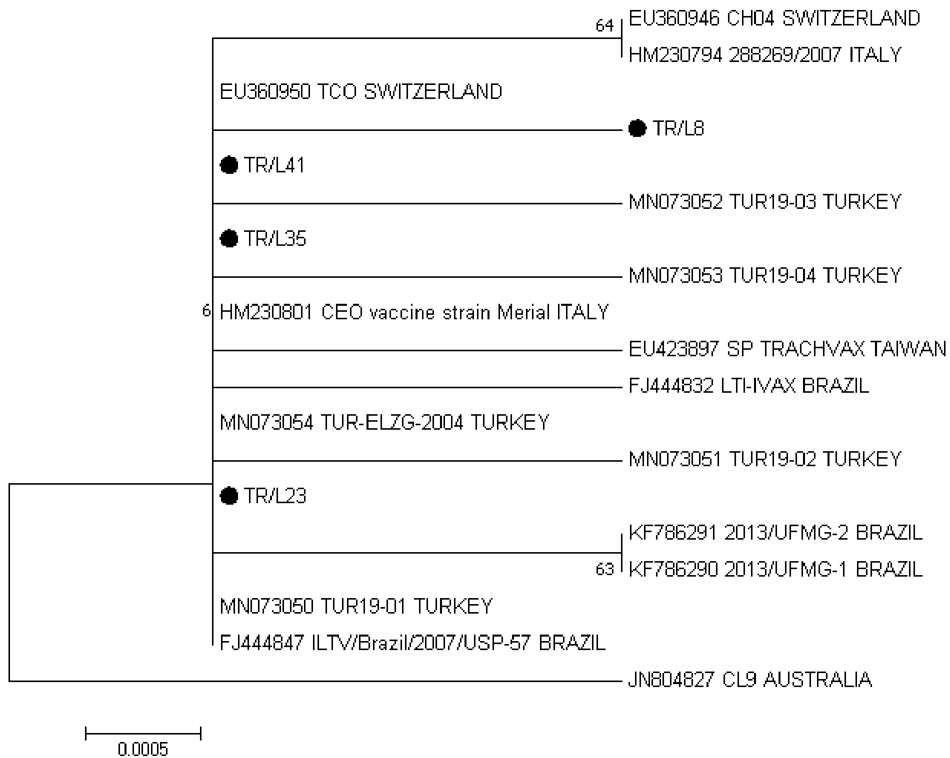

Figure 3. Phylogenetic tree regarding a partial sequence of the thymidine kinase (*TK*) gene, which indicates the relationship between the Türkiye strains and other infectious laryngotracheitis virus (ILTV) strains. This tree was generated using the neighbor-joining method with the Kimura 2-parameter substitution model and 1000 bootstrap replications. Türkiye isolates in the current study are indicated by black circles. The scale bar demonstrates the distance unit between sequence pairs. The sequences were acquired from GenBank.

revert to virulence during passage among vaccinated and contact birds. Hence, elucidating the origin of the virus involved in the outbreak is a crucial point that should not be overlooked. The extensiveness of the molecular characterization and differentiation of ILTV strains have been gradually increased, especially for epidemiological studies.

Avian respiratory disease has been known to be a complex issue that involves various pathogenic agents and environmental factors. Apart from IBV and ILTV, AMPV has been reported to be an important agent regarding its multifactorial, and mostly, subclinical characteristics. The complexity of avian respiratory disease has resulted in several difficulties and inadequacies concerning the confidential diagnosis of AMPV. Thus, molecular diagnosis of this agent has gradually increased its importance in poultry science worldwide. This study reports the detection of AMPV from Turkish broiler and layer flocks. The presence of AMPV was firstly reported by Bayraktar et al. [16] in Turkish broiler flocks. However, to the best of the authors' knowledge, there is no information about the prevalence of AMPV in layers; hence, this is the first study to highlight the presence of this virus in not only broilers but also layer chickens. Results of this study pointed out an unignorable prevalence (10.75%) in a total of 93 flocks. Moreover, a mildly higher frequency of AMPV was observed in layers (13.33%) compared to broiler flocks (8.33%). These results may emphasize the need for monitoring and in-depth knowledge of its spread in layer flocks. Although AMPV is a major frequent pathogen in turkeys, related infections have been reported worldwide in commercial chicken farms. In this context, the increased frequencies and the necessity of further studies have been previously reported by various researchers from Europe [29], Asia [30], Africa [31], etc. More importantly, the real prevalence of AMPV in Turkish flocks is still not known [16]. The lack of adequate molecular AMPV data prevents researchers from drawing any reliable conclusions and giving confidential suggestions. Taken altogether, the present study may provide important preliminary information about the AMPV prevalence in the Turkish poultry industry but comprehensive molecular studies may still be required.

In the present study, another virus that is known to cause significant losses to poultry farms is ARV. Although the most distinct symptom is arthritis in chickens diagnosed

with ARV-associated infection, the pathogenic strains can be isolated from chickens affected by assorted disease conditions, such as immunosuppression, gastrointestinal lesions, and acute or chronic respiratory diseases. ARV infections are mostly seen in many flocks worldwide, including Türkiye, and they are frequently encountered in broiler chickens and turkeys [32]. It is worth noting that comprehensive information on the prevalence of ARV infections is quite insufficient in Türkiye despite some previous studies conducted by serological analyses. The present study captured the baseline prevalence of ARV among broiler and layer flocks in Türkiye. Moreover, this study is the first report on the determination of ARV status in Türkiye by using molecular methods. Based on the σA-encoding gene analysis, the results indicated that 12.90% of all flocks (both broilers and layers) are infected with ARV. In addition, a remarkable high prevalence was found in broiler flocks (20.83%) compared to layers (4.44%). The results of the present research and previously published papers [33, 34] have shown that the ARV issue in the Turkish poultry sector has not been solved properly and the infections will continue to contribute to economic losses. Exhaustive molecular data sets are, therefore, required to achieve a better understanding of the etiology and to evaluate novel vaccine strategies and biosecurity procedures.

The subject of coinfections (also known as multiple infections, dual infections, or mixed infections) of respiratory diseases by different pathogens has received little attention even though such infections are common in poultry. Nevertheless, the presence of coinfections may cause alterations in the host-pathogen dynamics as part of the severity and duration of infection, host susceptibility, and pathology. In coinfected birds, primary pathogens can act as a predisposing factor for other pathogens. This situation may result in more severe clinical presentation in commercial poultry flocks. Here, we present the prevalence of the infections in combination with multiple pathogens including IBV, AMPV, and ARV. In this context, the frequency of both IBV+AMPV and IBV+ARV coinfections was 6.45% in total flocks. IBV+AMPV infection was found to be more frequent in layers (11.11 %), whereas IBV+ARV infection was more frequent in broiler flocks (10.42 %). On the other hand, only two broiler flocks were infected with triple virus infection (IBV+AMPV+ARV) and the corresponding rate was 4.17%. Such multiple infections can have an important impact on the development and severity of the diseases and should be taken into account in biosecurity practices and vaccination management in poultry.

The results of the present study demonstrate the complex epidemiology of respiratory tract viruses in layer and broiler flocks in Türkiye. Moreover, this study is the first report about a comprehensive epidemiological survey of multiple viruses and it provides important data on the status of coinfections of respiratory tract viruses in Türkiye. Thus, the molecular information provided by this study may be valuable not only for future studies on molecular epidemiology and the evolution of novel genotypes but also for current applications about improved diagnosis in the poultry industry.

Conflict of interest

No potential conflicts of interest were reported by the authors.

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Ethical statement

All procedures were performed complied with worldwide ethical considerations to ensure careful attention to animal welfare. Ethical approval was received from the Ethics Committee of Bursa Uludağ University (approval number: 2016-02/06).

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Table S1. List of selected IBV and ILTV strains used in the phylogenetic analysis.

Virus	Gene	Definition	GenBank accession number	Country
IBV	*S1*	TR8	KP259312	Türkiye
		AvCoV/chicken/TR/B19/trachea/2014	KT021473	Türkiye
		D1456/1/5/10_EG	KU238174	Egypt
		D1344/2/4/10_EG	KU238171	Egypt
		ACoV/teal/Benisuef-Egypt/VRLCU-11/2016	MF034382	Egypt
		IB VAR2-06	JX027070	Israel
		IS/1494/06	HM131453	Israel
		IS-1494	MG233398	Iran
		50_IBV_Romania_2017	MF101747	Romania
		gammaCoV/Ck/Poland/G052/2016	KY047602	Poland
		M41	AY561711	USA
		4/91 pathogenic	AF093794	UK
		4/91 vaccine	KF377577	
		Moroccan-G/83	EU914938	Morocco
		QXIBV	KC795604	China
		CK/CH/LDL/08III	GQ258308	China
ILTV	*ICP4*	TR2-70-1	MH921826	Türkiye
		TUR19-04	MN073045	Türkiye
		TUR19-03	MN073044	Türkiye
		TUR19-02	MN073043	Türkiye
		25/H/88/BCK	EU104899	USA
		CEO vaccine	EU104900	USA
		TCO vaccine	EU104908	USA
		24/H/91/BCK	EU104910	USA
		12/D/02/BCK	EU104911	USA
		USDA	JN542534	USA
		WangGang	DQ995291	China
		2012/UFMG-2 clone 1	KF786294	Brazil
		2013/UFMG-1 clone 1	KF786295	Brazil
		2013/UFMG-2 clone 1	KF786296	Brazil
		CL9	JN804827	Australia
ILTV	*TK*	TUR-ELZG-2004	MN073054	Türkiye
		TUR19-01	MN073050	Türkiye
		TUR19-02	MN073051	Türkiye
		TUR19-03	MN073052	Türkiye
		TUR19-04	MN073053	Türkiye
		2013/UFMG-1	KF786290	Brazil
		2013/UFMG-2	KF786291	Brazil
		LTI-IVAX	FJ444832	Brazil
		ILTV/Brazil/2007/USP-57	FJ444847	Brazil
		288269/2007	HM230794	Italy
		CEO vaccine strain Merial	HM230801	Italy
		SP-Trachivax	EU423897	Taiwan
		CH04	EU360946	Switzerland
		TCO	EU360950	Switzerland
		CL9	JN804827	Australia
		GaHV-1	NC_006623	USA
		WG	JX458823	China

IBV: Avian Coronavirus Infectious Bronchitis Virus; ILTV: Infectious Laryngotracheitis; *S1*: Spike 1; *ICP4*: Infected-cell protein 4; *TK*: Thymidine kinase