Pathological and molecular findings of visceral gout caused by Israel variant 2 (IS/1494/06) genotype of infectious bronchitis virus in chickens

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ABSTRACT

The aim of this study was to investigate pathological lesions and the presence of of Israel variant 2 (IS /1494/06) genotype of infectious bronchitis virus (IBV) in chickens with visceral gout. Sudden deaths were observed in broiler breeders and layer hens belonging to two different flocks located in eastern Türkiye. Broiler chickens were previously vaccinated with a vaccine containing strains of IBV Massachusetts serotype, but no information was available about the vaccination history of laying hens. At necropsy, there was accumulation of white chalk-like material on the serosal surface of the heart, liver, spleen and air sacs. The kidneys were unilaterally or bilaterally enlarged and pale in color and, ureters were also enlarged. Pooled organ samples from diseased chickens and kidneys alone were examined by virus isolation, real-time reverse transcription polymerase chain reaction (rRT-PCR), nucleotide sequencing analysis and histological methods. Israel variant 2 genotype of IBV was detected in the samples of both flocks as a result of virus isolation, rRT-PCR and DNA sequencing analysis. Histological examination revealed multifocal and randomly distributed crystal deposits in the renal tubulus and adjacent interstitium. Mild to moderate crystalline deposits surrounded by heterophils and macrophages were detected in the serosal layers of the heart, spleen, liver, and air sacs. The findings of this study indicated that IBV should be taken into consideration in visceral gout cases of chickens, and detection of IBV genotypes in the field will enable us to use vaccines compatible with these genotypes in order to control the disease more efficiently.

Introduction

Gout is a disorder characterized by increased uric acid in the blood and the accumulation of urates in organs due to impaired renal function (15). The disease is not seen in mammals except humans and anthropoid apes, because uric acid, the end product of purine metabolism, is converted to allantoin by the uricase enzyme. Owing to the absence of uricase enzyme in poultry, conversion of uric acid to allantoin, which is water soluble, does not occur which makes these animals prone to gout (39). In healthy poultry, uric acid binds with a specific protein in the proximal tubules of the kidney that prevents it from crystallizing in the kidneys, but disruption of kidney functions may lead to the occurrence of gout in birds (9, 28, 39). There are two forms of gout known as visceral gout and articular gout. Articular gout is rare in birds and has little economical significance (15). On the other hand, visceral gout is a multifactorial disease and many factors that cause kidney damage and impaired kidney functions may play role in the etiology of visceral gut. In visceral gout, urates accumulate on the serous surface of visceral organs such as the kidneys, heart, liver, spleen,

mesenterium, air sacs and peritoneum. Atrophy in one or both kidneys and dilatation in ureters with diffuse urate deposits may also be detected. Eventually, sudden and gradual deaths are seen in affected poultry (6, 7, 15).

The visceral gout has been linked with nutritional and toxic reasons as well as infections (9, 19). Among infectious agents, infectious bronchitis virus (IBV), avian nephritis virus (ANV) and chicken astrovirus (CAstV) have been showed to cause visceral gout (9, 10). IBV, the etiological agent of infectious bronchitis (IB), is an enveloped, single-stranded, positive sense RNA virus and takes place under Gammacoronavirus genus, subfamily of Coronavirinae and Coronaviridae family (20). IB is a highly contagious disease that occurs worldwide and causes huge economic losses in the poultry industry (17). The spike (S) protein of the virus has two subunits called as S1 and S2, the former provides the binding of the virus to the receptor while the latter performs the fusion between the virus and the cell membrane. Serotype and genotype classification is made depending on the changes in the S1 protein which shows the highest variability among the structural proteins (16). The S1 protein gene has multivariable regions responsible for the production of neutralizing and serotype specific antibodies (13, 27). The IBV has a large number of serotypes and new variants emerge due to frequent point mutation and recombination events in the genome of the virus. Although serotyping of IBV isolates is carried out by haemagglutination inhibition and virus neutralization tests (2), molecular methods have found widespread use in the diagnosis of the disease in recent years. Genetic identification of IBV is performed by replicating the S1 protein gene region by reverse transcription polymerase chain reaction (RT-PCR) and DNA sequence analysis (24).

In spite of the fact that the most effective method in controlling the disease is vaccination, the lack or insufficiency of cross protection against infections caused by genotypes different from the vaccine strains used in the field makes the control of IB difficult (36). Therefore, determining the genotypes of field isolates is very important in terms of monitoring new variants and evaluating vaccination programs. In Türkiye, there are a limited number of studies on frequency of IBV genotypes, in particular Israel Variant 2 (IS-var 2) which is nephropathogenic. Also, the role of nephropathogenic IBV in the etiology of visceral gout cases is overlooked. This study was therefore carried out to investigate pathological lesions and the presence of IS-var 2 genotype of IBV in broiler and laying hens with visceral gout.

Materials and Methods

Samples: Three dead and two live chickens from a broiler flock in addition to two dead chickens from a layer hen flock (totally five deaths and two live chickens) were submitted to the Department of Pathology at Fırat University located in Elazığ province. There were a total number of 850 chickens of Isa-Tinted breed at the average age of 18 weeks and the mortality rate was approximately 7% in the laying hen flock. No information was available on whether the animals had previously been vaccinated with IBV vaccine. In the broiler flock, there were a total number of 35.000 chickens of Ross 308 breed at the average age of 8 weeks and the mortality rate was approximately 6%. Animals in this flock were vaccinated with a vaccine containing strains of IBV Massachusetts serotypes. Sudden deaths were reported in both flocks. Pooled organ samples (kidney, heart, liver, spleen, sinus, air sac, larynx, trachea, lungs and genital organs) in addition to solely kidney samples collected from necropsied chickens were examined for the presence of IBV genotypes at the diagnostic laboratories of Bornova Veterinary Control Institute, İzmir.

Necropsy and histopathological examination: Systemic necropsy was performed in submitted dead and live chickens euthazised by servical dislocation under ether anesthesia, and tissue samples of kidneys, heart, liver, spleen, sinus, larynx, air sacs, lungs, trachea, cecal toncils and genital tract organs were collected, then fixed in 10 % neutral formalin solution. After processing routine procedures, prepared paraffin blocks were cut into 5 µm thick, stained with hematoxylin and eosin (H&E) and evaluated by light microscopy.

Virus isolation: Each chicken was assessed separately for virus isolation. Pooled organs and kidney samples were mixed with Phosphate Buffer Saline (PBS) (Sigma-Aldrich) containing penicillin (2000 units/ml), streptomycin (2 mg/ml) and gentamicin (50 µg/ml) antibiotics and, Mycostatin (1000 units/ml). The organs were homogenized using a MagNA Lyser (Roche) according to the manufacturer's instructions, followed by centrifugation at 3000 rpm for 10 min. The supernatants passed through a 0.45µm filter membrane were used for virus isolation and screened by real time reverse transcription polymerase chain reaction (rRT-PCR). 0.2 ml of the supernatants were inoculated onto the chorioallantoic cavity of ten 9-11 day-old specific pathogen free (SPF) eggs and were incubated at 37°C. Inoculated eggs were checked twice daily and those that died within 24h after inoculation were removed. Deaths between 2 and 7 days post inoculation (PI) were accepted as virus specific. The chorioallantoic fluid was picked up aseptically from embryos died between 48 and 72h PI, providing that the fluid did not demonstrate Hemagglutination (HA) activity. Dead embryos were investigated for the occurrence of embryo stunting, curling, urate in the mesonephros, or focal necrosis in the

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liver. Also, five live embryos taken from the incubator on day 3 PI were kept at 4°C for 24h and, the chorioallantoic fluids belonging to the embryos were used for the next passage (2, 33).

RNA extraction and cDNA synthesis: The chorioallantoic fluids collected on day 4 PI were serially diluted and used in Reverse Transcription-Polymerase Chain Reaction (RT-PCR). High Pure Viral Nucleic Acid Kit (Roche) was employed to extract total RNA from 200 µL of the chorioallantoic fluids according to the manufacturer's instruction. The extracted RNA was stored at -40 °C until PCR was performed. Viral RNA was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) and the obtained cDNAs were stored at -20°C until use.

Real-time reverse transription polymerase chain reaction (rRT-PCR): The rRT-PCR was performed on LightCycler480 (Roche, Mannheim, Germany) by using the Kylt IB-aCo Kit for the detection of Avian Coronaviruses, and the Kylt IBV-Variant O2 Kit for the detection of IBV Middle-East GI-23 lineage (Var2-like). The rRT PCR tests were conducted according to the manufacturer's instruction (AniCon Labor, Hoeltinghausen, Germany).

Partial sequencing of S1 gene: The cDNA samples detected as positive for IBV by rRT-PCR were subjected to partial sequencing of S1 gene by using two pairs of primers SX1: CACCTAGAGGTTTGYTWGCATG and SX2: TCCACCTCTATAAACACCYTTAC; SX3: TAATACTGGYAATTTTTCAGATGG and SX4: AATACAGATTGCTTACAACCACC (4). The first pair of primers (SX1 and SX2) were selected for use in the initial PCR and the other pair (SX3 and SX4) for nested PCR. First round amplification was performed in a final volume of 20 µL (2 µL D.W, 13 µL Norgenbiotek 2X PCR master mix (Canada), 2 µL of SX1 and SX2 primers and 3 µL of cDNA) with a thermal profile of one step denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 15 sec, 58 °C for 30 sec, 72 °C for 30 sec, and a final step of synthesis at 72 °C for 10 min. Amplifications were performed in an Eppendorf master cycler gradient thermocycler (Eppendorf, Hamburg, Germany). Nested-PCR reactions (total volume: 20 uL) were performed using 1 µL of the first PCR product. The reaction mixture was the same as the abovementioned PCR with the addition of nested primers (SX3 and SX4). The amplification products were analyzed by electrophoresis in 1.5% agarose gels in Tris-Acetate-EDTA (TAE) buffer, stained with GelRed™ (Biotium, USA) and visualized under UV light (33).

Nucleotide sequencing, alignment analysis and phylogenetic tree: The rRT-PCR products were purified and sequenced by ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, USA) in a forward direction using primer SX3 and in a reverse direction using primer SX4. The sequences obtained (345 bp) were compared with the IBV sequences in GenBank database and similarities were analyzed by BLAST. Multiple sequence alignments were carried out with Clustal W and phylogenetic tree was constructed with MEGA software (version 7; Biodesign Institute, Tempe, USA) using the neighbour-joining method and Tamura-Nei model with 1000 bootstrap (40).

Results

Gross findings: There was a diffuse accumulation of interspersed, white chalk like material on the serosal surface of the heart, liver, spleen and air sacs (Figure 1A). The kidneys were often markedly enlarged unilaterally or bilaterally with some atrophied lobes, pale in color, and the ureters were enlarged containing a white semi-fluid content that gave a cord-like appearance to ureters (Figure 1B).

Histopathological findings: There were similar histopathological findings in all cases. In kidneys, multifocally and randomly distributed radiating crystalline accumulations were seen in renal tubulus and adjacent intersititium, surounded by mild heterophils and macrophages (Figure 1C). In some areas, tubules exhibited acutely necrotic with degenerated or intact heterophils, while other tubules had eosinophilic protenous casts in the lumen. Renal intersitium contained prominent hemorrhage and congestion in addition to multifocal, moderate inflammatory infiltration including lymphocytes and macrophages (Figure 1D). Severe tubular dilation with flattened epithelium were observed. There were multiple fibrosis and mononuclear cell infiltratins in the wall and surrounded the ureters. Serosal layers of the heart, spleen, liver and air sacs showed crystalline depositoins surrounded by mild to moderate heterophils and macrophages. Myocardium adjacent epicardium also included mononuclear cell infiltrations and oedema, congestion, and myocardial degeneration/ necrosis. Trachea exhibited mild tracheitis characterised with mild mononuclear cell infiltrations in the propria mucosa, and the epithelium was intact (Figure 1E). In lungs, there was multifocal, mild mononuclear cell infiltrations localised in perivascular areas, and pleura was thickened due to moderate lymphocyte infiltrations (Figure 1F). There were no significant histopathological findings in the other organs.

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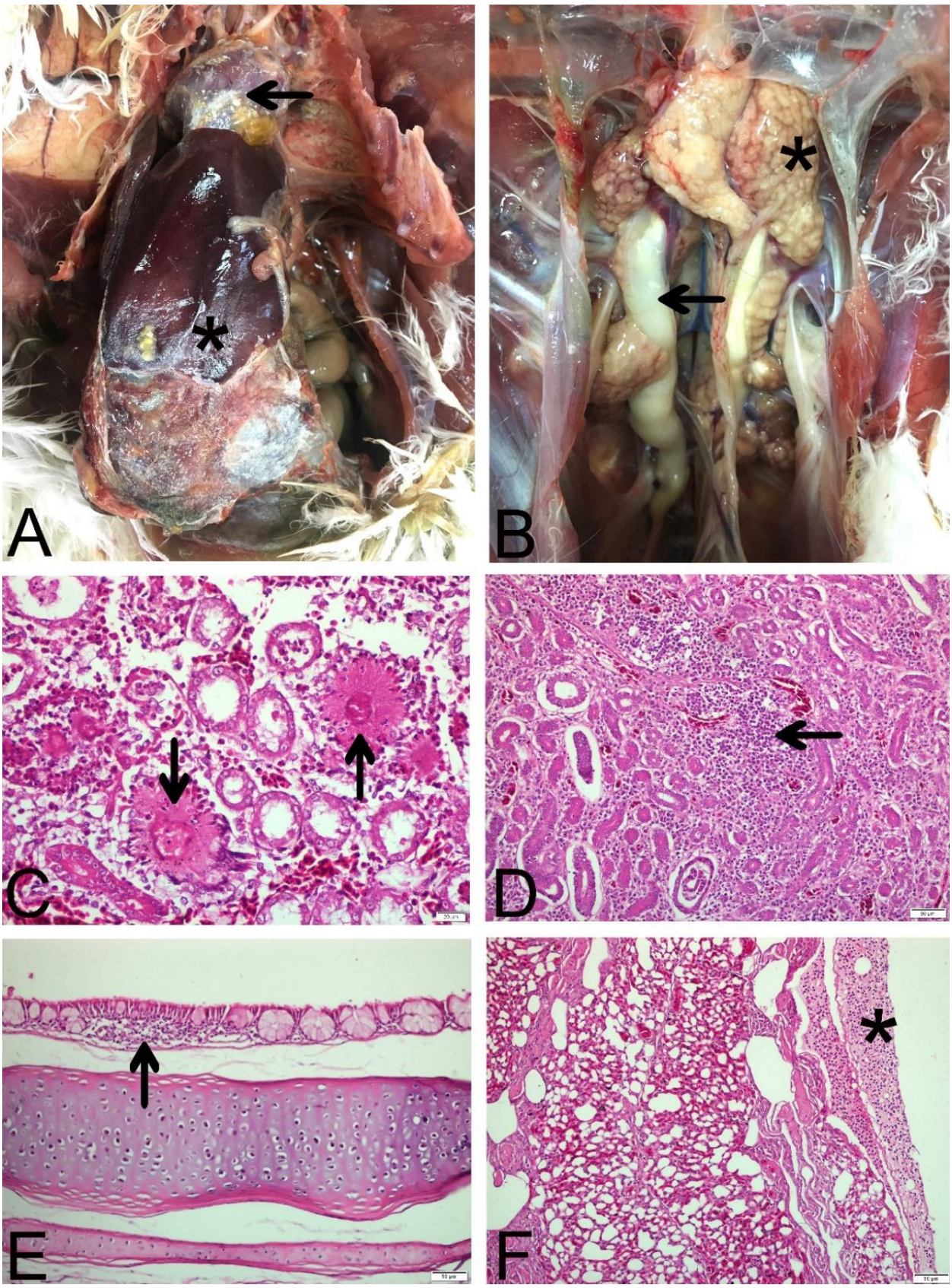

Figure 1. A. White chalk like material accumulations on the serosal surface of the liver (asterix) and heart (arrow). B. Enlargement and paleness in kidneys (asterix), enlarged ureters with a cord-like appearance (arrow). C. Radiating crystalline accumulations in renal tubulus (arrows), H&E staining, Scale bar = 20 µm. D. Intersititiel nephritis characterized with moderate inflammatory infiltration including lymphocytes and macrophages (arrow), H&E staining, Scale bar = 50 µm. E. Tracheitis characterised with mild mononuclear cell infiltrations (arrow) in the propria mucosa, H&E staining, Scale bar = 50 µm. F. Thickened pleura due to moderate lymphocyte infiltrations in lung (asterix), H&E staining, Scale bar = 50 µm.

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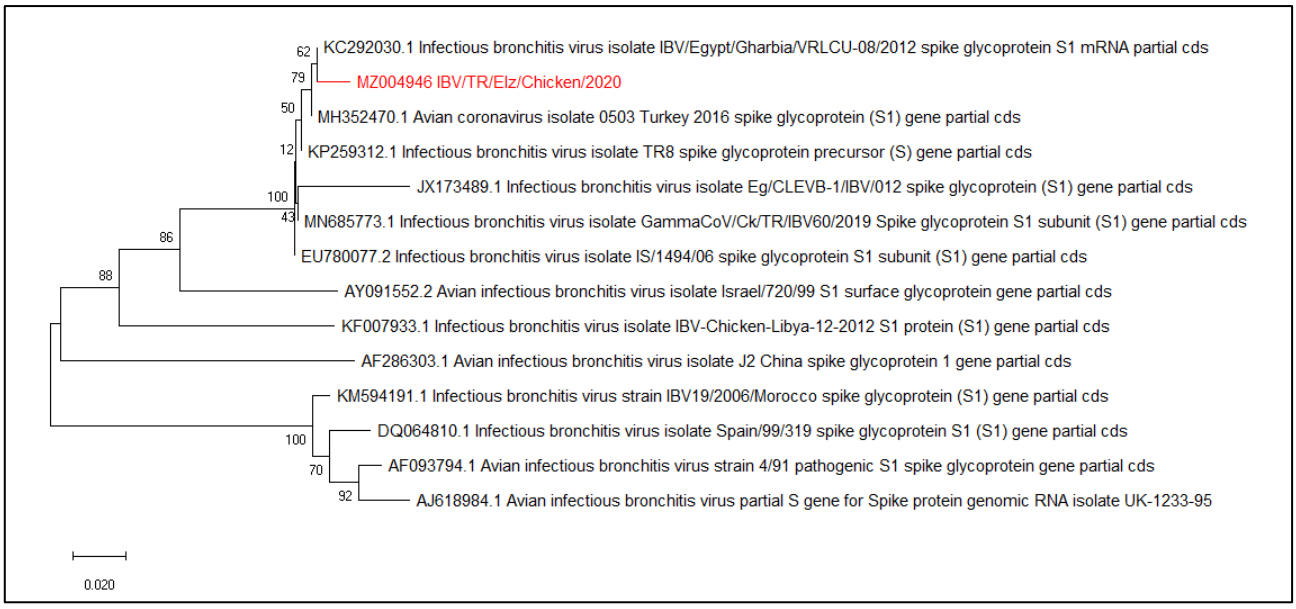

Figure 2. The phylogenetic tree of the sample determined as IBV/TR/Elz/Chicken/2020 (Accession number: MZ004946) constructed by neighbor-joining method.

Egg passage: After the seventh passage in SPF embryonated eggs, no specific lesions for IBV such as stunting, curling and uric acid deposition in the kidneys and ureter were observed. The chorioallantoic fluid of inoculated eggs were found to be negative for Newcastle disease virus and Avian Influenza virus by Hemagglutination (HA) Test.

rRT-PCR: The rRT-PCR analysis of homogenized organ samples showed positive results, with a threshold cycle value of 29.97 for Avian Coronaviruses, 32.70 for IBV Middle-East GI-23 lineage (Var 02). But, analysis of the infected chorioallantoic fluid samples could not yield any positive results.

Nucleotide sequencing, alignment analysis and phylogenetic tree: The results of S1 gene sequencing belonging to two isolates showed great similarity (approximately 98-99 %) with IBV IS-var2 (Accession No: MT270490.1). The phylogenetic tree of the sample determined as IS-Var2 (IBV/TR/Elz/Chicken/2020-GenBank Accession Number: MZ004946) was constructed by using the neighbour-joining method and Tamura-Nei model. Our isolates were detected to belong to genotype I lineage 23 (GI-23) based on the phylogenetic analysis (Figure 2).

Discussion and Conclusion

This study was carried out to describe patohological and virological findings of gout cases detected in chicken flocks. Gout can develop due to non-infectious and infectious causes. Non-infectious factors such as high calcium and protein diets, excessive salt, sodium bicarbonate intoxication, administration of high doses of gentamicin, vitamin A and D deficiency, imbalance between Ca-P levels, dehydration, mycotoxins and some administrative stress factors (9, 15, 38) can cause gout in poultry. In the present study, commercial ration and automatic watering regime were applied to the chickens in both flocks. When the feed composition was examined, it was observed that the protein, Vitamins A and D, calcium and phosphorus ratios in the ration were within normal levels. Mycotoxin analysis was not performed in feeds, but pathological examination revealed no findings specific for mycotoxicosis such as enlargement and paleness of liver, fatty degeneration, vacuolar degeneration, bile duct hyperplasia, fibrosis and enlargement of the bile duct (1). In addition, nephrotoxic antibiotics such as gentamicin were not administered to the animals in these poultry flocks. All these data suggested that non-infectious causes did not play a role in the occurrence of gout in chickens sampled in the present study.

IBV and astroviruses are considered among the infectious causes of gout (10). In this study, the samples were not examined in terms of astroviruses which were reported to cause disease mainly in turkeys (44). However, some astroviruses have been linked with enteric and kidney diseases in chickens (37). A few days/weeks old chickens have been reported to be more susceptible to astrovirus infection, and the disease was usually observed in animals up to 4 weeks of age (11, 23). Also, it has been reported that astrovirus can be found in healthy chickens (11). The average age of the chickens sampled here was 18 weeks for laying hens and 8 weeks for broilers. Also, due to the reports of previous studies conducted in Türkiye

that IBV infections were very common in chickens of all ages (25, 33, 43), gout cases in two flocks were examined for the presence of IBV infection only, in the current study.

IBV can cause lesions in the respiratory, urinary, genital and digestive systems in general (16, 17). Recently, nephropathogenic IBV has been detected in birds with visceral gout (19, 24, 30). In infections caused by nephropathogenic IBV, respiratory system is initially affected followed by severe kidney infection (36). In the present study, kidney enlargement, ureter dilatation, and uric acid crystals in the kidney, liver, heart, spleen and air sac were detected at necropsy. Microscopic examination revealed mild mononuclear cell infiltrates in the trachea and lungs, as well as multifocal and randomly distributed crystal accumulations in the renal tubulus and adjacent interstitium. Mild to moderate heterophiles and crystalline deposits surrounded by macrophages were observed in the serosal layers of the heart, spleen, liver, and air sacs. These findings suggested that some IBV strains involved in visceral gout had a strong affinity (tropism) to the kidney. Kidney damage caused by hyperuricemia can cause urates to accumulate on the surface of different visceral organs (39). According to the findings of this study, it is plausible to suggest that some IBV strains can disrupt kidney function and cause visceral gout by substantially proliferating in renal epithelial cells. In an experimental study investigating the reason for kidney affinity of IBV strains, nephropathogenic (B1648) and respiratory system pathogenic (Massachusetts-M41) IBV strains were compared and unlike the M41 strain, B1648 strain was found to grow better in peripheral blood monocytes cells and spread from the blood to internal organs (36). Nonstructural proteins such as Nsp1 and Nsp11, which are encoded by the ORF 1a gene in the genome of the nephropathogenic IBV, have been put forward to be responsible for nephropathogenicity (35). It has been determined that key genes in kidney tissue were associated with nephropathogenic IBV infection (42). However, additional studies are required to fully understand the kidney affinity (tropism) mechanism of some IBV strains.

Virus isolation requires several passages in embryonated eggs until stunting, curling or other signs are detected in the embryos (45). In the present study, the homogenized organ samples were positive by rRT-PCR for IBV. However, the virus could not be isolated from the samples and specific lesions for IBV were not detected in the embryos despite several passages. The failure of isolation might be due to the virus being inactive during the storage or transport of samples to the laboratory (45). In the present study, IS-var 2 genotype of IBV was detected in the examination of both pooled and kidney samples collected in two flocks with gout cases. The sequencing of hyper variable region (HVR) of the S1 gene (2) revealed that our isolates had similarity at about 98-99% with IS- var2-like isolates (IS/1494/06). The isolates were detected to belong to GI-23 lineage based on the phylogenetic analysis. Recently, GI-23 lineage has been reported to be widespread in many countries including Türkiye (22, 25, 33, 43). The first IS-var 2 IBV causing respiratory and nephropathogenic lesions was detected in Israel in 2004 (31). Later, this genotype has been reported in Lebanon, Libya, Jordan, Egypt and other countries in the Middle East (3, 5, 25, 32, 34). Although Mass41, 4/91, D274, Italy 02 and QX IBV are considered as the most common serotypes in Europe (12, 14), IS-var 2 genotype has been detected in recent studies conducted in European countries (18, 29, 41). The presence of this variant was reported in laying and broiler flocks in Türkiye (25, 33, 43). The determination of IS-var 2 genotype of Middle East origin in Türkiye and many other countries suggested that this genotype has spread to different continents through various sources. Uncontrolled human movements, animal tradeship and wild birds might be responsible for the spread of the disease between countries and continents (25). The fact that wild birds can carry Gammacoronaviruses asymptomatically may result in the emergence of new pathogenic IBV strains in poultry (26). Despite this, the use of live vaccines against a previously undetected strain in a region is not recommended, because possible genetic diversity of the virus can cause emergence of new strains due to recombination between field and vaccine strains (24). In Türkiye, it is known that Massachusetts (Ma5, M41, H120) and 793/B (4/91) serotypes vaccines are commonly used in chickens (43). In the present study, there was no information about vaccination history of the laying hen flock. On ther other hand, animals in the broiler flock were vaccinated with vaccines containing strains of IBV Massachusetts serotype, but no data were available on the protective antibody levels following vaccination. The finding of this study that IS-var 2 was detected in broiler chickens vaccinated against infectious bronchitis was similar to the results of previous studies conducted in different countries (5, 18, 32, 33). It is therefore suggested that the IBV vaccines widely applied in Türkiye do not provide cross protection against IS-var2 genotype. Significant difference between nucleotide sequences of S1 gene encoding immunogenic antigen of IS-var2 IBV detected in the current study and those of IBV strains within the vaccines widely used in Türkiye might explain inefficiency of the vaccines against infections caused by this genotype. However, it has been reported that live vaccines containing H120, D274 and QX IBV genotypes provided 50-70% protection against IS-Var2 IBV infection (8). Also in an experimental study, cross protection against IS-var2 (IS/1494/06) genotype was provided in chickens vaccinated with the combination of

H120-H120 and H-120-1/96 (793/B like) strains, though a complete protection was not noted (21).

It was concluded that nephropathogenic IBV should be considered in visceral gout cases of poultry. The fact that new variant strains may emerge depending on the changes in the genetic structure of the virus urges conducting large scaled epidemiological studies toward investigating variant strains circulating in both domesticated and wild birds. This will enable more effective and up to date vaccines against IS-var 2 IBV genotype to be included in poultry vaccination programs in Türkiye.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

NT, HO and HK conceived and planned the experiments. HO, HK, BK and FC carried out the experiments. NT, HO and HK planned and carried out the simulations. NT, AC and BK contributed to sample preparation. NT, HO, HK, HE and BC contributed to the interpretation of the results. NT, HK and BC took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

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