Concurrent infection of Infectious Bronchitis Virus and *Mycoplasma gallisepticum* in a backyard poultry

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ABSTRACT

This study reports the co-existence of two S1 mutants of GI-13 (4/91-like) Infectious Bronchitis Virus (IBV) and *Mycoplasma gallisepticum* (MG) in a backyard poultry flock that had non-vaccinated 30 broiler chickens and four turkey pullets. Serum samples and tracheal swabs were taken from the chickens and turkey pullets showing respiratory signs. Serum antibody levels were measured using commercial ELISA kits against IBV, Avian Influenza Virus (AIV), Newcastle Disease Virus (NDV), Avian Metapneumovirus (AMPV), MG, *Mycoplasma synoviae* (MS), and *Ornithobacterium rhinotracheale* (ORT). Additionally, tracheal swabs were tested for AIV serotypes H5, H7, and H9, NDV, IBV, AMPV, MG, MS, *Pasteurella multocida*, *Avibacterium paragallinarum*, and *Bordetella avium* by circular amplification technology (CAT). Anti-MS, -IBV, -MG, -NDV, -AMPV, and -ORT IgG antibodies were detected in some chicken sera, while anti-NDV, -MG, -MS, and -ORT IgG antibodies were detected in turkey sera. All avian tracheal swabs were positive for MG. However, IBV was only detected in chicken tracheal samples tested by CAT. The IBV strains were genotyped by sequencing a part of the *S1* glycoprotein gene. The alignment analyses of two isolates showed 99.35% and 98.69% nucleotide similarities and 99.02% amino acid similarities with the 4/91 IBV vaccine and field strains. Two mutants showed 99.35% nucleotide and 100% amino acid sequence identity to each other. The turkeys and chickens in the flock had MG and MG/IBV co-infections, respectively. Consequently, the presence of mutants of 4/91 (GI-13) IBV genotypes and MG found in backyard poultry could be a potential epidemiological source for commercial flocks in poultry integrations.

Introduction

Infectious diseases in areas of intensive poultry production can easily spread if poultry houses encounter an infection, especially one capable of aerosol transmission. In intensive production, strict biosecurity regulations are followed, but there are numerous factors that defuse these biosecurity practices against infectious diseases, including insufficient immunity in poultry flocks, improper vaccinations, inadequate vector control, and the presence of infection sources around poultry production facilities (16). Backyard poultry can be a source of infectious diseases for chickens and turkeys in commercial poultry premises. These backyard flocks are mostly in the gardens of villagers and can easily be exposed to viruses which are generally carried by migratory birds (28).

Viral and bacterial respiratory infections and related pathological problems such as swollen head syndrome and infectious sinusitis are the most common problems in poultry (1, 24). Avian Influenza Virus (AIV), Newcastle Disease Virus (NDV), Avian Coronavirus Infectious Bronchitis Virus (IBV), Avian Metapneumovirus (AMPV), *Mycoplasma gallisepticum* (*M.gallisepticum*: MG), *Mycoplasma synoviae* (*M. synoviae*: MS), *Pasteurella multocida* (*P. multocida*), *Avibacterium*

paragallinarum (*A.paragallinarum*), and *Bordetella avium* (*B. avium*) are responsible for respiratory tract infections in both chickens and turkeys, as well as some other birds such as finches, ducks, geese, pigeons (26). Some agents such as MG and MS can infect birds without clinical signs and cause sub-clinical infections. These agents can be easily transmitted vertically or laterally from bird to bird. Sub-clinical MG infections can show clinical chronic respiratory disease in chickens and infectious sinusitis in turkeys (7, 30). Additionally, birds infected with MG and MS are more prone to getting infected with other viral and bacterial agents such as IBV and *Escherichia coli* (18). The presence of IBV genotypes in backyard chickens has been reported in some studies. For example, in the United States, IBV was found to be the most commonly detected virus in backyard poultry between 2015 and 2017 (5). Moreover, Shokri et al. (25) demonstrated the presence of 793/B, IS/1494/06, and QX IBV genotypes in backyard poultry flocks as a potential source of IBV infection in commercial chicken flocks. In Canada, Brochu et al. (4) conducted a prospective 2-year prevalence study between 2015 and 2017 and reported that IBV was detected at a rate of 39% in all samples. They also stated that the most common co-infection was a combination of IBV, MG, and MS.

Backyard poultry production should be considered a great risk for a great number of poultry stocks in intensive poultry production units and commercial poultry production. Although this issue is very important in national poultry breeding, there is rather limited information on the infection dynamics of backyard production in the literature. Moreover, few studies have focused on avian respiratory diseases at the molecular level in poultry production. In this study, we investigated the possible viral and bacterial respiratory agents and demonstrated a co-infection consisting of the 4/91 IBV genotype and MG in a non-commercial backyard poultry flock where chickens and turkeys were being bred together within the same garden. We then genetically characterised the two mutant IBVs that were detected in the 4/91 genotype of the GI-13 genetic lineage from chickens.

Materials and Methods

Sampling: The study was performed on a backyard flock located in the Bandırma district of the province of Balıkesir, Türkiye. This flock was in the center of the province of Bandırma where numerous commercial poultry production companies are located. The backyard flock consisted of 30 broiler chickens and 4 turkey pullets. All chickens and turkey pullets showed severe respiratory symptoms such as swollen head, swollen submaxillary sinus, gasping, as well as dullness, fatigue, and depression. None of these animals had been vaccinated against any poultry infections. Blood samples and tracheal swabs were taken from four turkey pullets and randomly selected 12 chickens. Blood samples (1.5-2 ml) were drawn from *Vena cutanea ulnaris* into 2 ml sterile microfuge tubes. Sera were obtained by centrifugation at 1300× g for 10 min and stored at −20 °C until analysis. Twelve chicken and four turkey pullet tracheal samples collected using sterile swabs were pooled to comprise four individual swabs each, and used as four samples for subsequent analysis. For this, tracheal swabs were pooled and placed in sterile tubes containing phosphate-buffered saline (PBS). After vortexing vigorously for 15-20 seconds, each swab was pressed against the inner wall of the tube to release the collected material into PBS and was stored at -20°C for further tests.

ELISA for antibody detection: To detect AIV-, NDV-, IBV-, AMPV-, MG-, MS-, and ORT-specific IgG, commercial ELISA kits were used. The test procedures were performed based on the instructions provided by the manufacturers (for AIV-, NDV-, IBV-, AMPV-, MG-, MS-specific IgG: BioCheck, UK, Ltd., London, United Kingdom; for ORT-specific IgG: IDEXX, Westbrook, ME, USA). Absorbance was measured at 405 nm (for AIV-, NDV-, IBV-, AMPV-, MG-, and MS-specific IgG) and 650 nm (for ORT-specific IgG) using a BioTek ELx800 ELISA Reader (BioTek Instruments Inc., Winooski, USA). The cut-off values were used as given in the kit instructions.

Nucleic acid extraction: Tracheal swabs were transferred to Molecular Transport and Lysis Reagent (MTRL) tubes (Nucleogene Biotechnology Co., Istanbul, Türkiye) and left for 30 min. All liquids were then transferred to a spin column placed in a collection tube, and they were centrifuged at 8000xg for one minute. 500 µl of 80% ethanol was added into the spin column and centrifuged at 8000xg for one minute. Next, the spin column was centrifuged for one minute at 16000xg until there was no residual ethanol left. Afterward, 50 µl of Nuclease-Free Water was added to the center of the spin column and centrifuged at 8000xg for one minute. Finally, the obtained nucleic acids were stored at -80°C.

Nucleic acid detection: To detect possible viral and bacterial etiological agents, we used circular amplification technology (CAT) (Nucleogene Biotechnology Co., Istanbul, Türkiye) based on the principles of the isothermal amplification of nucleic acids (11). We performed the CAT method to detect a specific part of the genes of AIV serotypes H5, H7, and H9, NDV, IBV, AMPV, MG, MS, *P. multocida*, *A. paragallinarum*, and *B. avium*. The working principle of the CAT test is based on the binding of 10 specific primers and 3 special

enzymes to a targeted gene region. Ten primers bind to the targeted gene region, and these primers then fold into DNA or RNA that has been translated into cDNA using3 special enzymes to form loops. By introducing primary radiation marked with a dye to these loops, they are measured with a Molecular Detection Assay device, curves are drawn on the screen, and positive samples are determined (23).

IBV genotyping: A nested reverse transcriptase polymerase chain reaction (RT-PCR) described by Worthington et al. (29) was modified to genotype the IBV isolates. The first round of amplification was carried out in a final volume of 25 µl of a mixture containing 0.5 µl of the primers SX1+ (CACCTAGAGGTTTGT/CTA/TGCAT) and SX2- (TCCACCTCTATAAACACCC/TTT), 2.5 µl of RNA, 5 µl OneStep RT-PCR Buffer (Qiagen), 1 µl of OneStep RT-PCR Enzyme Mix (Qiagen), 1 µl of dNTP mix (Qiagen), and 14.5 µl of ddH_2O. For the second nested PCR, a mixture at a volume of 20 µl [0.4 µl of the primers SX3+ (TAATACTGGC/TAATTTTTCAGA) and SX4- (AATACAGATTGCTTACAACCACC), 10 µl of Taq PCR master mix (Qiagen), 7.2 µl of ddH_2O, and 2 µl of the first amplicon] was prepared. The first thermal profile was set up as 50°C/30 min for the RT stage, 95°C/15 min, 30 cycles at 94°C/10 min, 50°C/1.5 min, and 72°C/2 min in order. The second profile was set up as 94°C/3 min, 30 cycles at 94°C/1 min, 48°C/1.5 min, and 72°C/2 min in this order using a thermal cycler (BioRad C100 Touch Thermal Cycler, BioRad Laboratories, California, USA). The amplification products were purified and sequenced using an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer). We evaluated the similarities between the detected IBV samples and previously reported IBV genotypes in the BLASTn online tool provided by NCBI based on partial *S1* sequences. Multiple sequence alignments were performed using Jalview v2.7. Next, the aligned sequences were subjected to bootstrapping (1000 replicates), and a phylogenetic tree was constructed with MEGA 7 v7.0.26 by the neighbor-joining method with a Kimura two-parameter model (17).

Results

ELISA results: Among the chicken serum samples (n=12), 83.33% (n=10) had MS-specific IgG, 50% (n=6) had IBV-specific IgG, 33.33% (n=4) had MG-specific IgG, 16.67% (n=2) had NDV-specific IgG, 16.67% (n=2) had AMPV-specific IgG, and 8.33% (n=1) had ORT-specific IgG. However, all samples were negative for AIV (Table 1). The IgG levels for each chicken sample are presented in Figure 1. The rates of dual, triple, and quadruple antibody presence were found in 33.33%, 25%, and 8.33% of the samples, respectively. All turkey samples were positive against NDV and MG. Additionally, 75% (n=3) had MS-specific IgG, and 25% (n=1) had ORT-specific IgG. None of the turkey samples had AIV-, IBV-, or AMPV-specific IgG (Table 1).

Table 1. Numbers and percentages of chicken and turkey serum samples with positive IgG OD levels for AIV, NDV, IBV, AMPV, MG, MS, and ORT.

	Chicken (n=12)		Turkey (n=4)	
	n	%	n	%
IBV	6	50	0	-
MG	4	33.33	4	100
MS	10	83.33	3	75
AMPV	2	16.67	0	-
ORT	1	8.33	1	25
NDV	2	16.67	4	100
AIV	0	-	0	-

Molecular detection of agents: The chicken samples were found to be negative for AIV serotypes H5, H7, and H9, NDV, AMPV, MS, *P. multocida*, *A. paragallinarum*, and *B. avium*, while they were positive for MG and IBV. The turkey samples were found to be positive for MG. Some examples of amplification curves for the positive and negative samples are presented in Figure 2a-2d.

We then identified the genotypes of the two IBV isolates detected according to the *S1* gene sequence analysis. The BLAST analysis revealed that two isolates (AvCoV/backyard/chicken/Bandirma1/21 and AvCoV/backyard/chicken/Bandirma2/21) were closely related to the 4/91 variant (GenBank Acc. No: AF093794). The nucleotide similarity rates of AvCoV/backyard/chicken/Bandirma1/21 and AvCoV/backyard/chicken/Bandirma2/21 to the original 4/91 IBV (AF093794) were 99.35% and 98.69%, respectively. The amino acid similarity rate between the 4/91 strain and the two isolates obtained in this study was 99.02%. Moreover, AvCoV/backyard/chicken/Bandirma1/21 and AvCoV/backyard/chicken/Bandirma2/21 showed 99.35% nucleotide and 100% amino acid sequence identity to each other (Figure 3a, 3b). Two mutations were observed at positions 801 (thymine to cytosine) and 1062 (adenine to thymine) in AvCoV/backyard/chicken/Bandirma2/21 compared to the 4/91 *S1* sequences. There were also two mutations at positions 1092 (thymine to adenine) and 1095 (thymine to adenine) in AvCoV/backyard/chicken/Bandirma1/21 and AvCoV/backyard/chicken/Bandirma2/21. The mutations in the nucleotide sequence observed in our IBV isolate led to three silent mutations (amino acid positions 267, 354, and 364). On the other hand, the mutation at amino acid position 365 was a missense mutation recognised by the phenylalanine to leucine alteration.

To determine the phylogenetic relationships among the IBV strains, we analysed the *S1* gene sequences. As shown in Figure 4, two IBV strains in our study were clustered into GI-13 genotype that includes 4/91 vaccine (KF377577), 4/91 pathogenic strain (AF093794), UK/2016/81 (MH590028), AvCoV/chicken/TR/L37/2017 (OL956527), and CR88-UPM2013 (KM067900). Two IBV strains obtained in this study were deposited to GenBank with the following access numbers: AvCoV/backyard/chicken/Bandirma1/21 (OL981643) and AvCoV/backyard/chicken/Bandirma2/21 (OL981644).

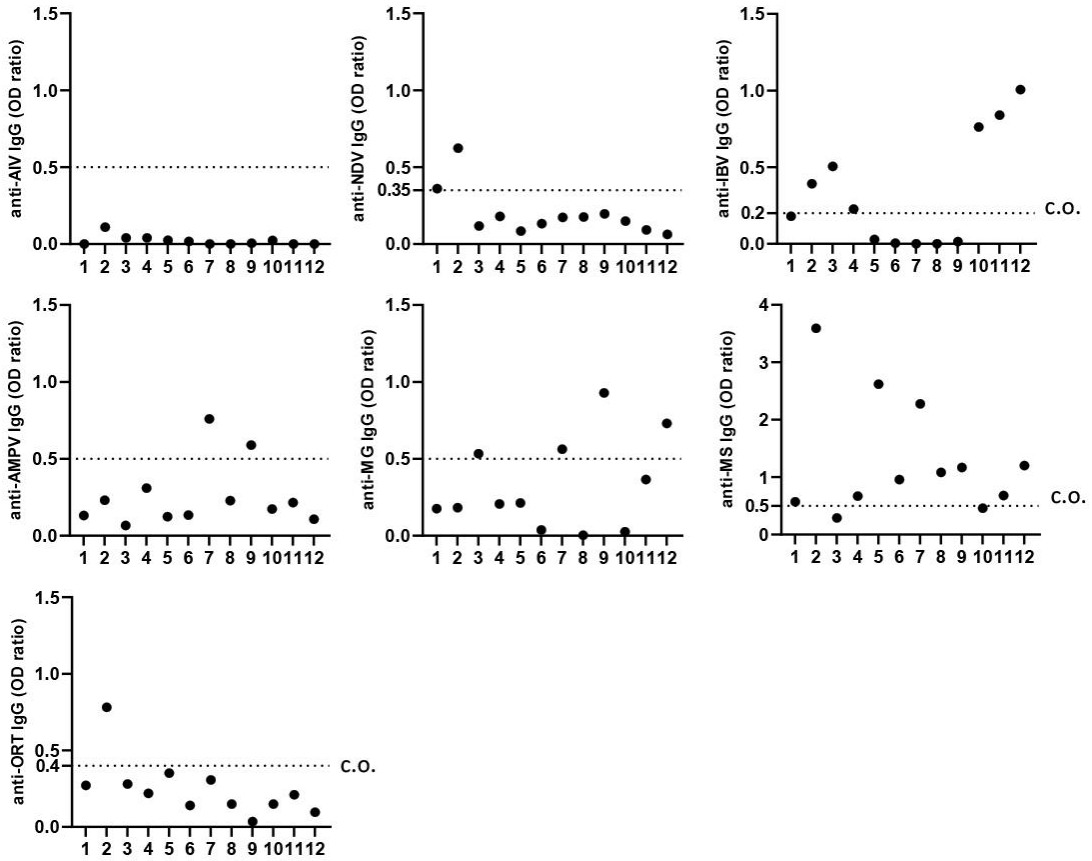

Figure 1. AIV, NDV, IBV, AMPV, MG, MS, and ORT-specific IgG levels in chicken samples. Horizontal lines indicate cut-off values (c.o.) given the instructions of the kit.

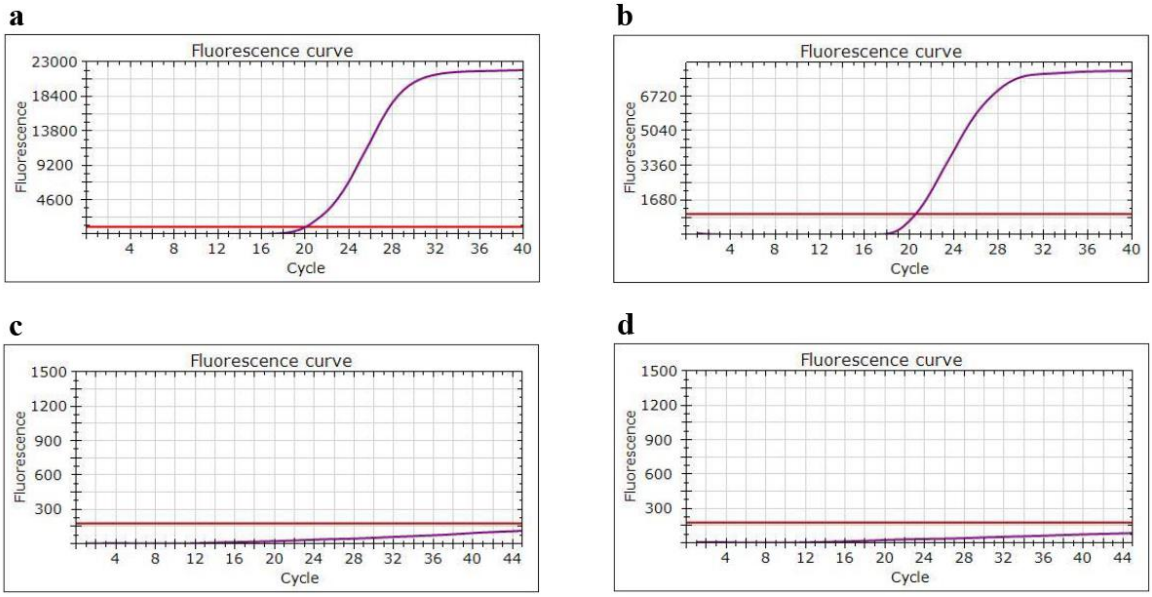

Figure 2. Results of circular amplification technology. Graphic demonstration of positive amplification curves for (**a**) MGand (**b**) IBV and negative curves for (**c**) NDV and (**d**) AMPV.

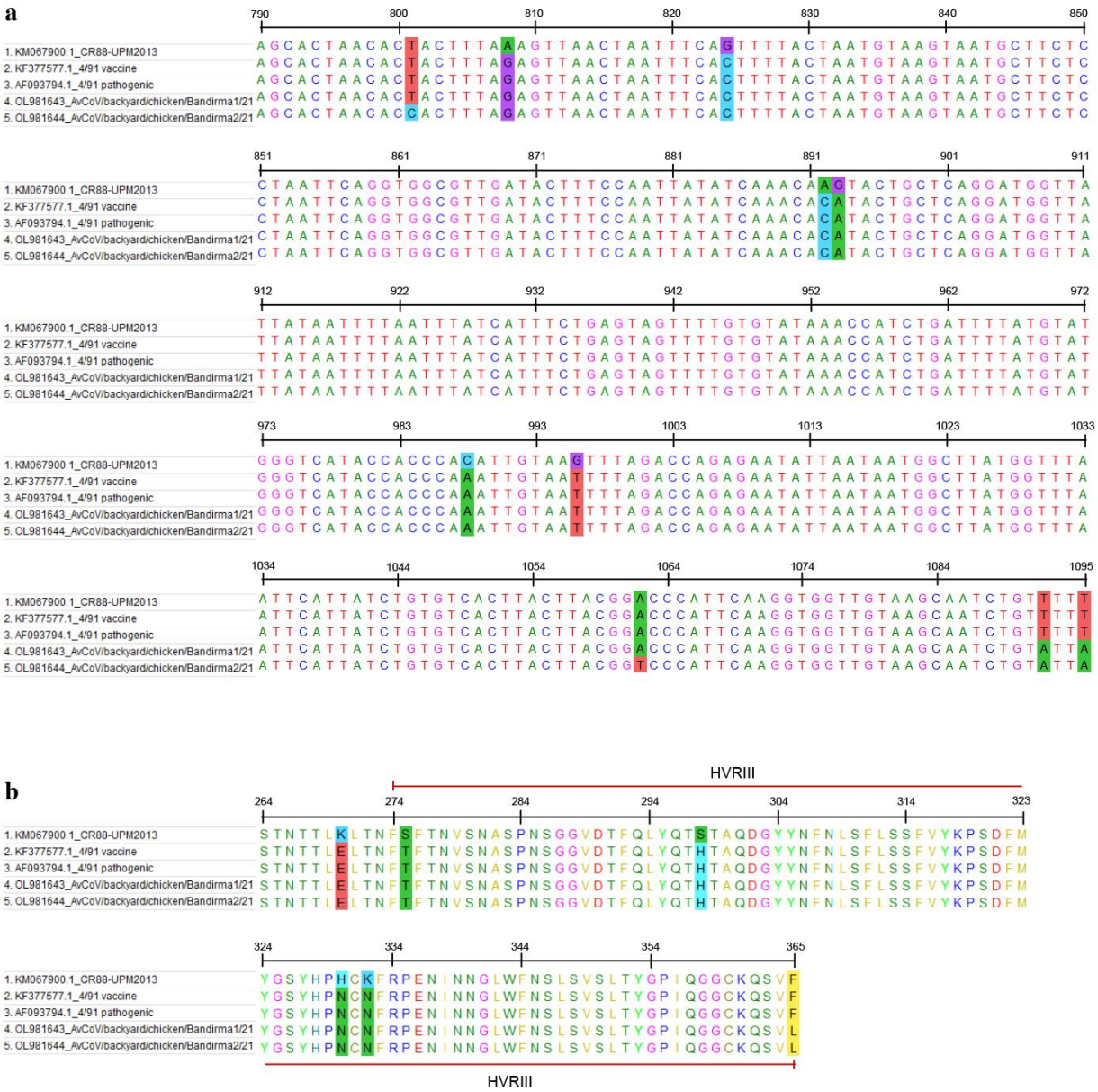

Figure 3. Alignment and comparison of a partial *S1* sequence. (**a**) Nucleotide and (**b**) amino acid sequences of *S1* gene comparison of two IBV isolates (AvCoV/backyard/chicken/Bandirma1/21 and AvCoV/backyard/chicken/Bandirma2/21) obtained in this study. HVRIII: hypervariable region III.

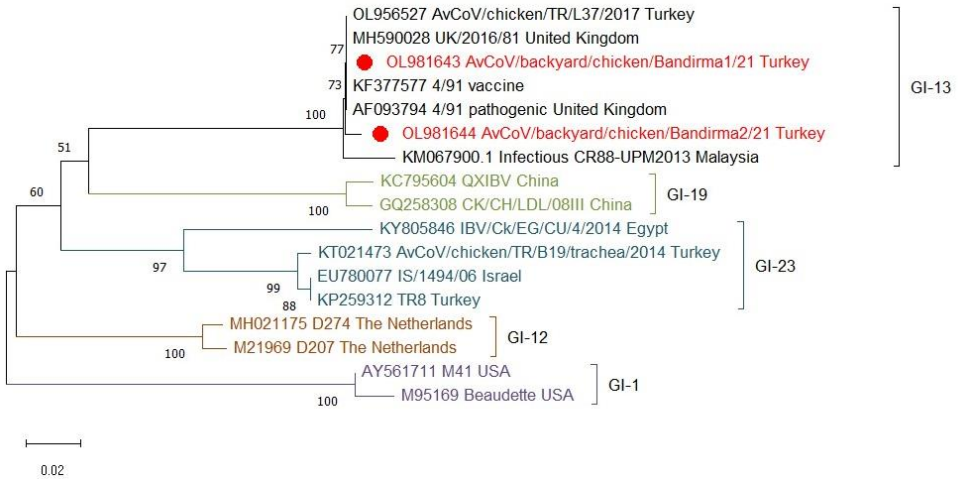

Figure 4. Neighbour-joining tree constructed using the Kimura two-parameter model based on partial sequence of the *S1* gene sequences.

Turkish isolates in the current study are indicated by red circles. The scale bar demonstrates the distance unit between sequence pairs. The sequences were acquired from GenBank.

Discussion and Conclusion

The presence of common avian viral and bacterial respiratory pathogens such as IBV, AIV, NDV, AMPV, MG, and MS in backyard poultry as single infections or co-infections may cause a great threat to commercial chicken flocks. Backyard flocks, especially those that are not immunised with live or inactivated virus vaccines, can be infected with field strains of pathogenic microorganisms more easily. Hence, they become reservoirs for pathogenic bacteria and viruses for commercial chicken flocks (4, 5, 7). Therefore, in this study, we screened the tracheal samples of chickens and turkeys showing respiratory signs for the presence of AIV serotypes H5, H7, and H9, NDV, IBV, AMPV, MG, MS, *P. multocida*, *A. paragallinarum*, and *B. avium*. We found that the samples of the chickens had MG and IBV, while the samples of the turkeys had only MG. Additionally, anti-IBV and anti-MG IgG ELISA antibody titers in the serum samples were also a supportive result, indicating active infections with IBV and MG in the chickens in the studied flock (Figure 1). We also observed that most of the serum samples had high antibody titers to MS, although MS could not be detected in the tracheal samples. This may suggest that most antibody positivity for MS infection could be possible because of cross-reactions (false positives) frequently seen in serological tests (8, 19). Anti-MS antibodies observed in this study could also be a result of the high sensitivity of the ELISA kits which may have led to cross-reactivities of other antibodies with non-pathogenic or other pathogenic mycoplasma species such as MG (9, 15). In terms of NDV, the ELISA results were positive for NDV-specific IgG, while tracheal swabs were negative based on the CAT method in our study, which was in agreement with the results reported by Marks et al. (20). This may be due to circulating NDV strains with low pathogenicity, which may induce seroconversion with inconspicuous clinical manifestations. Moreover, we could not detect IBV in the tracheal samples of the turkeys that had close contact with the IBV-infected chickens. A possible explanation is that IBV could not naturally infect turkeys because IBV and the turkey coronavirus (TCoV) are host-dependent avian coronaviruses. They only share ~34% similarity for the S protein sequence (13).

In our study, the presence of MG infection both in turkeys and chickens in the flock may have been a result of vertical or lateral transmission. In Türkiye, chicks and turkey pullets in small backyard flocks are sold in the open market for non-commercial purposes (6). These are mostly discarded unhealthy young birds, and they can be infected with pathogenic bacteria such as MG and MS (30). Additionally, the studied flock was being reared in a primitive and open-top garden which was open to exposure to humans and exotic wild birds that could be mechanical or biological carriers of MG. The co-existence of MG and IBV in commercial chickens with respiratory problems is frequently seen in the field and reported in several studies (10, 24). However, the existence of the dual infection of MG and IBVs in backyard poultry as a source of these infections for commercial poultry facilities here can be considered uncommon (4, 5) and valuable epidemiological data. These data revealed that preventive IBV and MG control attempts such as vaccinations seem to be an urgent need and should be implemented in backyard flocks grown near poultry production companies.

The genotyping of the IBV samples indicated that both isolates were assigned to the GI-13 lineage (27), and they shared nucleotide identities of 99.35% and 98.69% with both pathogenic and attenuated 4/91 IBV strains showing us that we had two different S1 mutants in these backyard chickens. Up to our knowledge, there is no previously published data regarding the detection of any genotype of IBV from backyard chickens in Türkiye although the presence of the genotypes in this genetic lineage in backyard chickens has been reported in several countries as Canada, United States, and Iran (4, 5, 25). Remarkably, IBV genotypes can vary regionally, and their prevalence in backyard poultry may differ from commercial settings. However, due to the lack of comprehensive studies on IBV genotypes particularly in backyard chickens worldwide, specific information about the prevalence and distribution of genotypes in this context is limited. On the other hand, there are a number of studies on the detection and genotyping of IBV from commercial chickens with respiratory problems in Türkiye. For example, the first genotyping and isolation study on IBVs in commercial poultry flocks was performed by Kahya et al. (14), and the isolate was designated as GI-23 (IS/1494/06). Another large-scale survey in Türkiye indicated that the heterogenicity of the IBV genotypes consisted of M41-based isolates in GI-1, 4/91, or 793/B-related isolates in GI-13 and IS/1494/06 isolates in GI-23 (31). In a recent paper, Mustak et al. (22) reported that IS/1494/06 is the most prevalent genotype in commercial broiler flocks. Moreover, these researchers indicated that H120, 793/B, and D274 genotypes are also circulating in Türkiye. Here, it should be noted that our ongoing laboratory studies and a recently published paper (2) on the genotyping of IBV isolates in broiler and layer chicken flocks showed that 793/B- or GI-13-related IBV detection rates in respiratory samples have increased, and 793/B IBVs have become the second most dominant genotype subsequent to IS/1494/06 IBVs. Taken together, the cumulating evidence has demonstrated that dominant IBV genotypes in commercial poultry are GI-13 (793/B) and GI-23 (IS/1494/06) in Türkiye (2, 22, 31). The beginning of the occurrence of 793/B IBV detections in

chicken flocks after 2016 in Türkiye might be a result of the usage of live-attenuated vaccines in chicken flocks although there were no infection problems with the same wild-type genotype in the field (31). The usage of novel vaccine strains has always been an infection source in the field worldwide because attenuated vaccine IBVs can regain their virulence (3).

In this study, we detected four mutations at the nucleotide positions of 801, 1062, 1092, and 1095 (Figure 3a). Among these, three were silent mutations. Nevertheless, the mutation at nucleotide position 1095 led to F/L alteration in amino acid position 365 (Figure 3b). This F/L mutation can be considered to cause critical conformational changes in the antigenic epitopic region located in the hypervariable region 3 of the S1 protein of IBV (12). Additionally, we examined the genetic connection of our two IBV isolates to the IBV vaccine strains of CR88, 4/91, and 4/91 field strains belonging to the GI-13 lineage based on the partial *S1* gene and amino acid sequences (Figure 3a, 3b). The phylogenetic analyses (Figure 4) of our two IBV isolates revealed that these isolates could be genetically closer to the 4/91 isolates than the CR88 isolate. A possible reason could be the widespread usage of 4/91-derived vaccines compared to CR88-derived ones in commercial chicken flocks in Türkiye.

While the sample size may be limited, it is important to emphasize that these samples are representative of a backyard setting lacking vaccines, drugs, and biosecurity measures. Additionally, the absence of similar backyards in the vicinity, coupled with the high density of commercial poultry flocks in the area, renders this sampling unique. Consequently, the present analysis was undertaken using this distinctive set of samples.

In conclusion, the IBV isolates that were examined in this study were closely related to 4/91 vaccine strains, which are commonly used in this region of Türkiye. The possible mutations of attenuated IBVs or the presence of native 4/91 field strains with mutations may have the potential to overcome the protective immunity induced by vaccine strains. Additionally, backyard chickens infected with such mutant IBV isolates can be evaluated as an important epidemiological source for the development and introduction of novel mutant IBVs for commercial chicken flocks. Apart from this, the existence of MG in the IBV-infected backyard chickens in this study could have been a result of the vertical transmission of the agent from breeder flocks or the horizontal transmission of the chickens by wild birds infected with MG, respiratory aerosols, hatchery transmission or indirect modes including environmental factors and fomites (21, 30).

We strongly recommend that backyard flocks, along with commercial chicken flocks, be monitored regularly and continuously for IBV genotypes. Furthermore, the screening of mutations in epitopic sites in the *S1* gene is a critical practice for evaluating the current knowledge about the presence of circulating IBVs and logically selecting the vaccine protectotype of IBV. Thus, the level of protective immunity against novel variants led by mutations, especially in the HVRs of the *S1* gene, can be increased in flocks.

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Conflict of interest

No potential conflict of interest was reported by the authors.

Author Contributions

ÖA, SKD, and KTC conceived and planned the experiments and took the lead in writing the manuscript. TSK, AGC, and AÖ contributed to sample preparation. TSK and SE carried out the experiments. ÖA and KTC contributed to the interpretation of the results. 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.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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