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Original Article

Concurrent Occurrence of Infectious Bursal Disease and Multicausal Respiratory Infections Caused by Newcastle Disease and Avian Metapneumovirus in Broilers

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Abstract

Control strategy of respiratory complex infections should address precipitating and predisposing causative agents in general and immunosuppressive agents in particular. In both clinical and subclinical forms, infectious bursal disease virus (IBDV) is one of the most immunosuppressive diseases of young chickens. This study aimed to investigate the concurrent occurrence of subclinical infectious bursal disease (IBD) and multicausal respiratory complex infections caused by Newcastle disease virus (NDV) and avian metapneumovirus (aMPV) in broilers. In this study, 800 tissue samples (e.g., trachea, cecal tonsil, bursa of Fabricius, and spleen) and 400 sera samples were collected from broilers with confirmed respiratory signs selected from 20 broiler farms in west Azerbaijan province, Iran, from October 2018 to February 2019. Pathogens in the tissue samples were detected using RT-PCR for the VP2 gene of IBDV, F gen of NDV, and N gene of aMPV. The amplified products were sequenced afterward. At the end of the husbandry period, sera samples were used to detect antibodies against IBDV, aMPV, and NDV using ELISA and HI tests. Molecular results showed that the 45% (9/20), 30% (6/20), and 15% (3/20) of tissue samples were positive for IBDV, NDV, and aMPV, respectively. Regarding co-infection, 5% (1/20) of farm isolates were positive for IBD and ND, while 10% (2/20) of farms isolates were positive for IBD and aMPV. Co-infection of IBD, ND, and aMPV was not detected in farm isolates. Serological results indicated that the IBD co-infected flocks had almost higher (P<0.05) antibody titers against IBD; however, IBDV-NDV co-infected flocks and IBDV-aMPV co-infected flocks had lower antibody titer against NDVand aMPV, respectively. It can be concluded that lower antibody titer against ND and aMPV in IBD-ND and IBD-aMPV co-infections indicated suppressive effects of IBD on these diseases. Therefore, vaccination against IBD even in regions without clinical form of IBD is inevitable for the reduction of immunosuppressive effects of subclinical IBD on immune responses against these diseases.

Keywords: Avian metapneumovirus, Broiler, Bursal disease virus, Newcastle disease, Respiratory complex

1. Introduction

The occurrence of respiratory co-infections due to the presence of multiple causative agents is more prevalent in poultry. Where the respiratory disease in poultry is clinically exacerbated, the precise diagnosis with an effective treatment becomes a challenge. Therefore, control strategies of respiratory complex infections should address both precipitating causative agents and predisposing factors (1, 2). Regarding predisposing parameters with suppressive effects, IBD is one of the most immunosuppressive avian pathogens of young chickens (3-5). On the other hand, among respiratory viral diseases, avian metapneumovirus (aMPV), as a single-stranded negative-sense RNA virus, is the most dominant pathogen in co-infections in broiler chickens (5, 6). Newcastle disease (ND) is endemic in Iran, and

many commercial poultry farms have been affected in recent years (7-10). Recent studies revealed that the occurrence of both clinical and subclinical forms of Gumboro disease had an immunosuppressive impact on chickens (11). It is well documented that the exposure of chickens to IBD viruses (IBDV) prior to vaccination could eliminate the protective effects of the vaccine (12). The immunosuppressive impact of IBDV varies based on its serotypes, strains (i.e., avirulent, classical, variant, and very virulent) of serotype 1 of IBD, and types of poultry productions (i.e., broilers, layers, and breeders) (13). Based on the evidence, the subclinical form of IBD, which occurs mostly in young chickens with inadequate maternally derived antibodies (14), could affect respiratory infections via two mechanisms (13). In the first mechanism, IBDV' antigens were found in the trachea as the main site for entrance and replication of aMPV and Newcastle disease virus (NDV) (15). In the second mechanism, IBDV mainly impaired the humoral immunity, and cellular and innate immunity were also being affected. Accordingly, in chickens exposed to IBDV, the immune responses to the routine vaccination are negatively affected (12). Therefore, the present study was designed to investigate the concurrent field occurrence of IBD, ND, and aMPV in broilers with respiratory complex infections.

2. Materials and Methods

2.1. Chickens

In total, twenty broiler farms ($10x10^3$-$30x10^3$ birds) with clinical signs of respiratory infection (i.e., sneezing, nasal discharge, coughing, foamy conjunctivitis, swollen infraorbital sinus, unusual increasing daily mortality) were selected from various regions in West Azerbaijan province, Iran, between October 2018 and February 2019. The studied flocks aged between 3 to 6 weeks.

2.2. Sampling

At least 20 broiler chickens with clear respiratory clinical signs of infection were humanely euthanized and autopsied in the first stage (2). A total number of 800 samples from the trachea, spleen, cecal tonsils and bursa of Fabricius were harvested from each flock as previously described (2, 13) to create an organ-specific pool (two birds/pool) for RNA extraction. Furthermore, on 45-50 days of age (at the end of the husbandry period), 400 blood samples (at least 20 samples/flock) were also taken from the brachial vein of chickens from the selected broiler flocks as previously described (2) to determine antibody titers against IBDV, NDV, and aMPV.

2.3. Molecular Characterization:

2.3.1. RNA Extraction

RNA of tissue samples was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The extracted RNA was divided into three equivalent parts and each part was used to detect IBD, ND, and aMPV viruses.

2.3.2. Reverse Transcription (RT) and Polymerase Chain Reactions (PCR)

Complementary DNA (cDNA) was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Burlington, Canada), according to the manufacturer's instructions. The synthesized cDNAs were used as a template for PCR to detect IBDV, NDV, and aMPV. The PCR conditions and primer pairs used for the molecular study of IBDV, NDV, and aMPV are presented in table 1 (16-18).

2.3.3. Sequence Analysis

After purification of positive PCR products (QIAquick PCR purification kit, Qiagen, Hilden, Germany), the amplicons were submitted for nucleotide sequencing using forward and reverse primers (Bioneer, South Korea). All fragments were sequenced in both forward and reverse directions using the Sanger dideoxy sequencing technology.

2.3.4. Phylogenetic Analysis

The nucleotide sequences of the hvVP2 gene of IBD, F gene of ND, and N gene of aMPV obtained in this study were subjected to BLAST (primary genotyping and similarity results), aligned and compared to reference strains downloaded from the NCBI's GenBank database. Sequence homology analysis was

performed using MEGA7.0. Phylogenetic trees were constructed with the Neighbor-Joining method in MEGA7.0 (bootstrap values of 1000) using the Kimura 2 parameter model (7).

2.3.5. Serological Tests

Blood samples were kept at room temperature (approximately 2 h at 37℃) until clotted and sera were collected afterward. It was then centrifuged using 1500 rpm as previously described (2), and stored at -70℃ for detection of antibody titers against IBDV and MPV, and against NDV using Elisa and HI test, respectively.

2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA test is the most commonly used method for the evaluation of antibodies against IBD and aMPV (4, 6). Sera were tested by indirect ELISA using commercial IBDV and aMPV ELISA kits (Flockchek, IDEXX Laboratories, Inc., USA) to determine IBDV and aMPV antibodies, according to the manufacturer's instructions. Optical density values were obtained at 650 nm wavelength on an ELISA reader (BioTek ELX800). Results were finalized through the calculation of the sample to positive (S/P) ratio as recommended.

2.5. Hemagglutination Inhibition (HI)

A HI test was performed to determine antibody titers against the NDV (10, 19).

2.6. Statistical analysis

The data were analyzed using SPSS software (Version 23.0; IBM Corp, Chicago, USA) employing one-way ANOVA. The means of different groups were compared with Bonferroni post-hoc test. Data were presented as the mean±standard error (SE). A *P*-value less than 0.05 ($P \leq 0.05$) was considered statistically significant.

Table 1. Primer sequences and PCR conditions used for detection of IBDV, NDV, and aMPV

Agent	Primers	Gene	(5'-3') Sequence	Size	PCR conditions			Ref.
				(bp)	Cycle	Time	°C	
IBDV	B3F	hvVP2	CCCAGAGTCTACACCATA	474	1	2 min	94	16
					35	20 s	94	
					35	25 s	50	
	B4R		TCCTGTTGCCACTCTTTC		35	30 s	72	
					1	5 min	72	
aMPV	Nd	N	AGCAGGATGGAGAGCCTCTTG	115	1	2 min	94	18
					40	30 s	94	
					35	30 s	50	
	Nx		CATGGCCCAACATTATGTT		35	1 min	72	
					1	5 min	72	
NDV	F	F	GGTGAGTCTATCCGGARGATACAAG	202	1	2 min	94	17
					35	35 s	94	
					35	35 s	50	
	R		TCATTGGTTGCRGCAATGCTCT		35	160 s	72	
					1	7 min	72	

IBDV: Infectious bursal disease virus, aMPV: Avian metapneumovirus, NDV: Newcastle disease virus

3. Results

Results of molecular detection of certain pathogenic agents are presented in table 2. Based on types (none, single, and multiple) of isolated viruses (IBDV, NDV, and aMPV), the broiler chicken farms were allocated to group 1 (negative field IBDV, NDV, and aMPV), group 2 (positive field IBDV, negative field NDV, and aMPV), group 3 (positive field NDV, negative field IBDV, and aMPV), group 4 (positive field aMPV, negative field IBDV, and NDV), group 5 (positive field IBDV and ND and negative field aMPV), and group 6 (positive field IBDV and aMPV and negative field NDV).

Table 2. Characteristics of 20 naturally infected flocks with respiratory complex infections

Flock No.	Age (day)	Mortality (%)	Virus	RT-PCR	Virus isolate	Elisa	HI
1	35	6.5	IBDV	+	IR/H2965-1/18	4503	-
			NDV	-	-	-	3.2
			aMPV	-	-	230	-
2	15	12	IBDV	+	IR/H2965-2/18	4967	-
			NDV	-	-	-	3.6
			aMPV	-	-	237	-
3	22	7	IBDV	-	-	4002	-
			NDV	-	-	-	3.8
			aMPV	-	-	260	-
4	25	8	IBDV	+	IR/H2965-4/18	6235	-
			NDV	-	-	-	2.9
			aMPV	-	-	222	-
5	27	6	IBDV	-	-	2838	-
			NDV	-	-	-	4.3
			aMPV	-	-	257	-
6	30	12	IBDV	+	IR/H2965-6/18	7841	
			NDV	-	-	-	2
			aMPV	-	-	217	
7	33	12	IBDV	+	IR/H2965-7/18	6492	
			NDV	-	-	-	3.1
			aMPV	+	IR/H12965-7/18	4552	
8	40	8	IBDV	-	-	3455	-
			NDV	-	-	-	2
			aMPV	-	-	255	-
9	42	5.5	IBDV	+	IR/H2965-9/18	6552	-
			NDV	-	-	-	3.9
			aMPV	-	-	234	-
10	39	7	IBDV	-	-	4622	-
			NDV	-	-	-	3
			aMPV	-	-	242	-
11	36	6	IBDV	+	IR/H2965-11/18	6452	-
			NDV	-	-	-	3.5
			aMPV	+	IR/H2965-11/18	3730	-
12	35	4	IBDV	-	-	4423	-
			NDV	-	-	-	4.1
			aMPV	-	-	256	-
13	26	7	IBDV	-	-	2330	-
			NDV	+	IR/H2963-13/18	-	6.1
			aMPV	-	-	256	-
14	35	10	IBDV	-	-	2575	-
			NDV	+	IR/H2963-14/18	-	6.8
			aMPV	-	-	252	-
15	29	8	IBDV	+	IR/H2965-15/18	6359	-
			NDV	+	IR/H2965-15/18	-	5.1
			aMPV	-	-	241	-
16	27	9	IBDV	-	-	1451	-
			NDV	-	-	-	2.5
			aMPV	+	IR/H2965-16/18	5832	-
17	22	8	IBDV	+	IR/H2965-17/18	5401	
			NDV	-	-	-	3
			aMPV	-	-	268	-

Flock No.	Age (day)	Mortality (%)	Virus	RT-PCR	Virus isolate	Elisa	HI
18	19	36	IBDV	-	-	2571	-
			NDV	+	IR/H2965-18/18	-	6.7
			aMPV	-	-	250	-
19	27	6	IBDV	-	-	2587	-
			NDV	+	IR/H2963-19/18	-	6.2
			aMPV	-	-	268	-
20	27	6	IBDV	-	-	1907	-
			NDV	+	IR/H2963-20/18	-	5.2
			aMPV	-	-	237	-

Phylogenic trees of IBDV, NDV, and aMPV are presented in figure 1, figure 2, and figure 3, respectively. Elisa antibody titers against Gumboro disease and avian metapneumovirus infection are presented in Figure 4 (A and C, respectively), and HI antibody titer against ND is presented in figure 4B. As shown in table 2, 45% (9/20), 30% (6/20), and 10% (2/20) of flocks were positive for IBDV, NDV, and aMPV, respectively. There has been no routine vaccination against aMPV in the Iranian broiler sector; therefore, all aMPV isolates (Figure 3) were considered as field isolates and the infected flocks had high antibody titers against aMPV (Figure 4B). Flocks having antibody titers ≤296 against aMPV were considered negative, according to IDEXX Elisa kit interpretation. Moreover, 7 out of 9 (77.7 %) of IBDV isolates and 2 out of 6 (33.3 %) of NDV isolates were field strains (Figure 1-2). Regarding co-infection, 1 out of 20 (5%) farms was positive for both IBDV and ND and 2 out of 20 (10%) farms were positive for IBDV and aMPV. Co-infections of NDV and aMPV and co-infection of IBD, NDV, and aMPV were not found during this investigation.

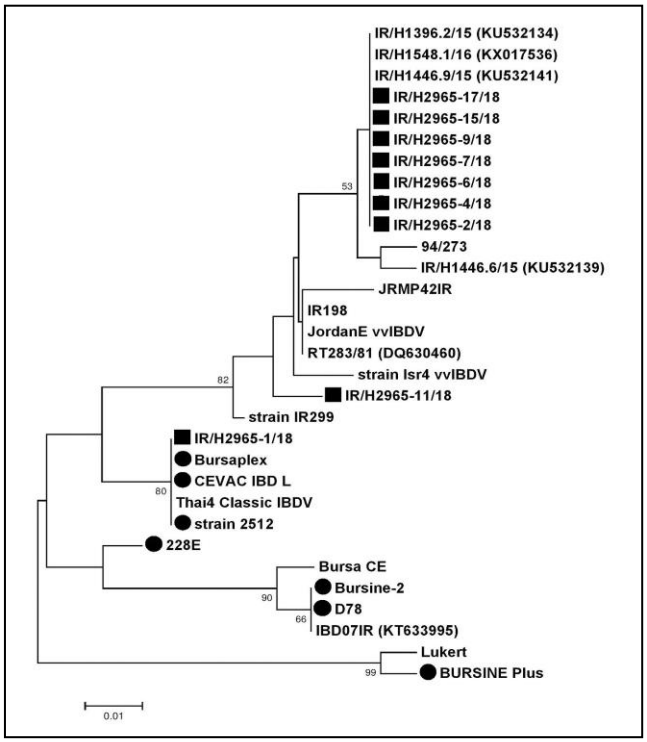

Figure 1. Phylogenetic analysis of the VP2 hypervariable coding sequence of 9 IBDV isolates. The viruses characterized in this study are indicated as a bold black square. The tree was constructed using the neighbor-joining method. Bootstrap values were based on 1,000 replications. The difference in the number of amino acids is indicated by the bar at the bottom of the Figure. The sequences were obtained from NCBI Gene Bank. The scale bar represents 0.01 substitutions per site.

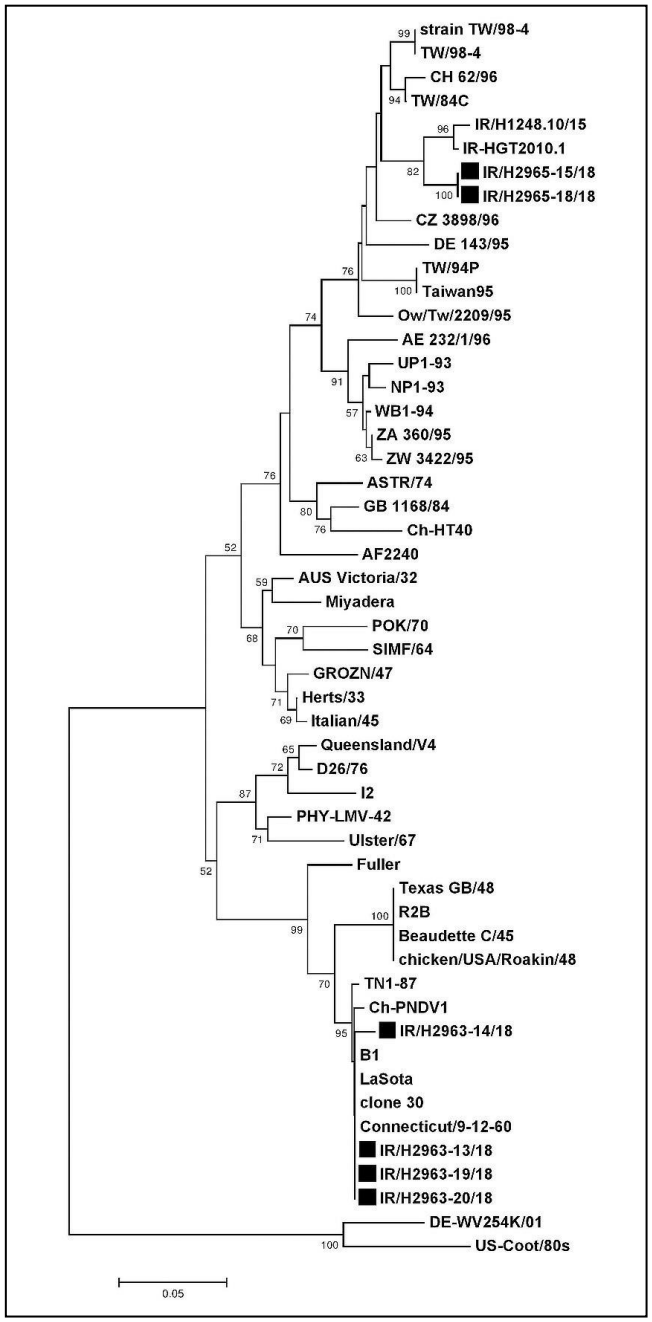

Figure 2. Phylogenetic analysis of the F gene coding sequence of 6 NDV isolates. The viruses characterized in this study are indicated as a bold black square. The tree was constructed using the neighbor-joining method. Bootstrap values were based on 1,000 replications. The number of amino acids difference is indicated by the bar at the bottom of the figure. The sequences were obtained from NCBI Gene Bank. The scale bar represents 0.05 substitutions per site.

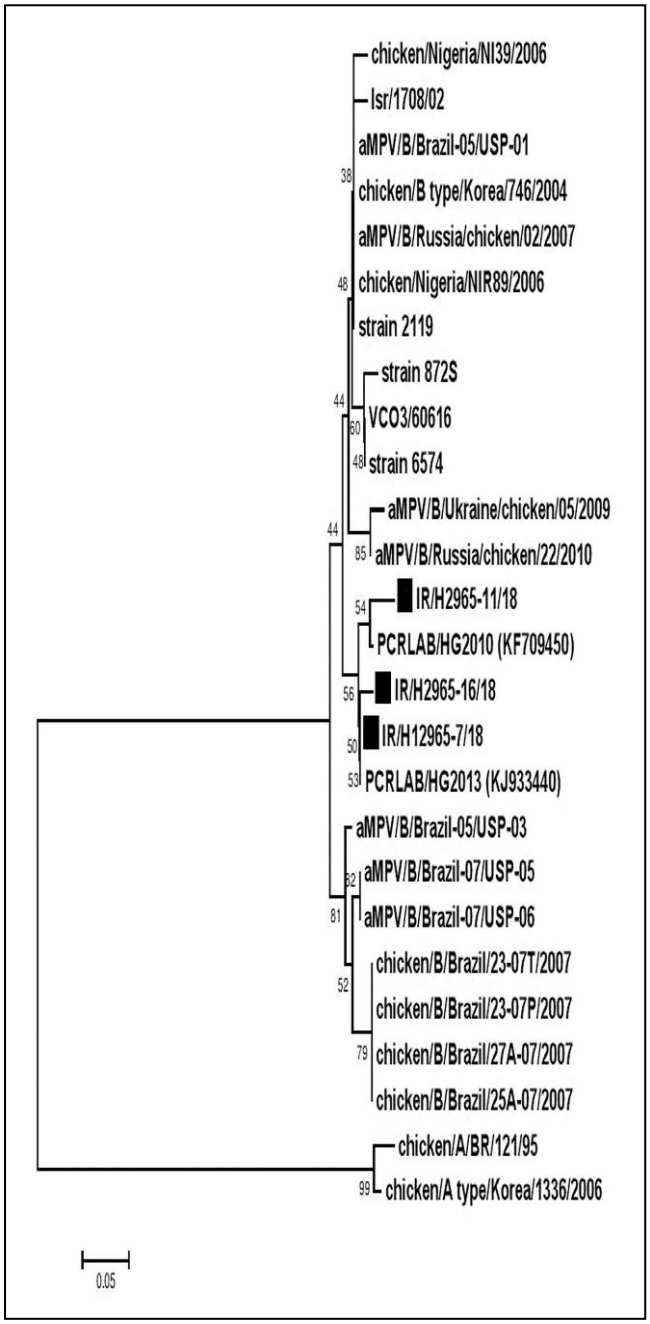

Figure 3. Phylogenetic analysis of the pair Nd/Nx primers of N gene coding sequence of 3 aMPV isolates. The viruses characterized in this study are indicated as a bold black square. The tree was generated using the Neighboring Joining model with MEGA. Bootstrap values were based on 1,000 replications. The difference in the number of amino acids is indicated by the bar at the bottom of the Figure. The sequences were obtained from NCBI Gene Bank. The scale bar represents 0.05 substitutions per site.

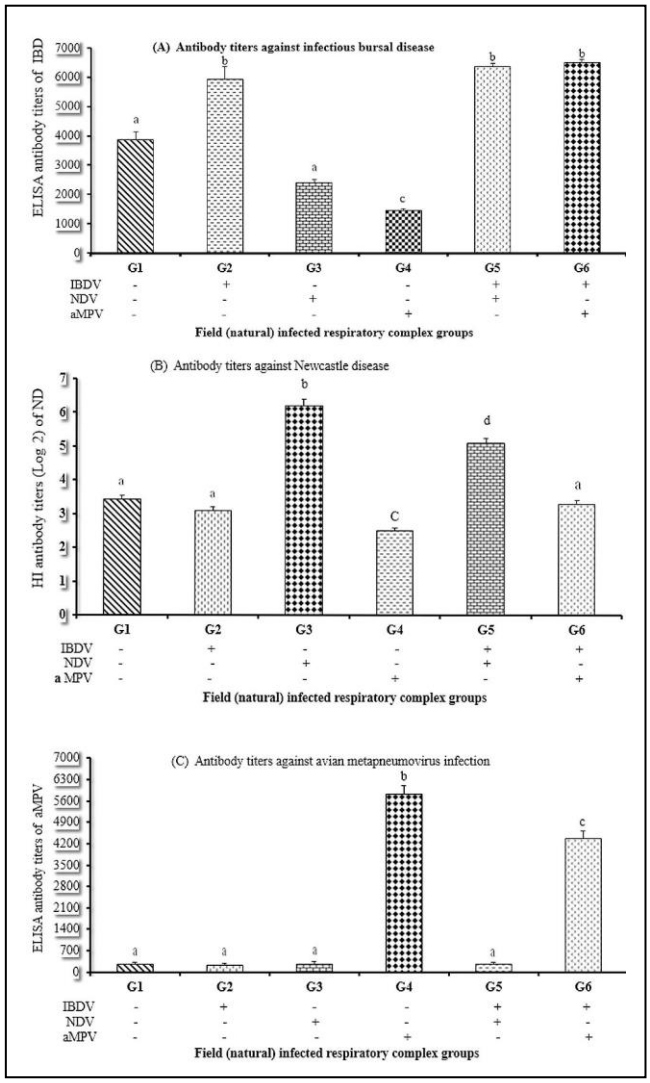

Figure 4. Antibody titers against IBD (A), ND (B), and aMPV (C) of naturally respiratory complex infected groups; G1 (field IBDV, NDV, and aMPV isolates negative farms), G2 (field IBDV isolates positive farms), G3 (field NDV isolate positive farms), G4 (field aMPV isolates positive farms), G5 (field IBDV and NDV isolate positive farms), and G6 (field IBD and aMPV isolates positive farms). Different superscript letters indicate significant differences between the groups (P<0.05).

4. Discussion

In this study, NDV, aMPV, and IBDV detected in broilers with respiratory complex infections are among the multiple pathogens present in complicated respiratory diseases as previously reported (20). Despite the presence of the pathognomonic signs of respiratory infection, the absence of IBDV, NDV, and aMPV (Table 2) from some studied flocks indicated the possibility of involvement of other respiratory pathogens, including avian influenza and infectious bronchitis viruses in respiratory complex diseases, as previously documented (21).

4.1. Infectious Bursal Disease (IBD)

The IBDV, especially in subclinical form, is still present in broiler farms and this observation is in agreement with previous reports, suggesting that the majority of field infections were the subclinical form of IBD (Table 2) (3, 14). Co-infections of IBDV+NDV and IBDV+aMPV were also observed in this study (Table 2), and co-infected flocks had antibody titers less than those with a single infection, confirming that the subclinical IBD affected immune responses to these diseases (Figures 4B and 4C). Considering the role of subclinical IBD as a predisposing and immunosuppressive factor in the occurrence/severity of respiratory infections, several mechanisms have been proposed in this regard. It has been reported that IBDV has a significant impact on gut-associated lymphoid tissues and the subsequent change in the microbiota (22). Furthermore, the localization of IBDV antigens has also been reported in the respiratory ciliated epithelium, indicating that IBDV may also exert its predisposing effects on the colonization of respiratory pathogen via damaging the trachea tissues as a site of entrance and replication of respiratory pathogens (5). Most importantly, both clinical and subclinical IBD are immunosuppressive (11, 12)and have major impacts on both humoral (by the destruction of IgM+ cells) and cellular (upregulation of cytokine genes by activated IBD virus-induced bursal T cells) immune responses against most of the avian pathogens involved in respiratory infections (13, 14). Based on the phylogenic analysis, seven out of nine IBDV isolated during this study were field isolates (Figure 1) and shared some common amino acid sequences of the segment A gene with hvIBDV isolates from Pakistan (Eastern neighbor); however, they were partly different from IBDV isolated from Western neighbor countries (Turkey and Iraq), indicating that IBDV has been in the

process of evolution in Iran. The results of the present study were in line with the findings of previous reports (23). Regarding antibody titers against IBD of co-infected flocks (Figure 4A) with IBDV and NDV as well as IBDV and aMPV, the obtained results showed that co-infected flocks (G5 and G6) had higher antibody titers, compared to flocks in IBDV infected farms (G2), indicating that NDV and aMPV had no negative effects on antibody against IBDV and even elevated it in some degrees.

4.2. Newcastle Disease Virus (NDV)

As presented in figure 2, two out of six (>33 %) NDV isolates were field isolates and previous studies showed that NDV circulating at the broiler industry in IRAN belonged to the sub-genotype VII.1.1 (VIIL) and were genetically very similar, having evolutionary distances of 0.0 to 0.7 from each other (7). One of the studied flocks (Table 2) had a single infection with NDV field isolate and 36% mortality rate, indicating the virulence of the isolated virus. As shown in figure 4B, antibody titers of NDV positive group (G3) were higher (P<0.05) than those of NDV negative groups (G1, G2, G4, and G6) as well as those of the co-infected group (G5), indicating that isolates significantly induced higher humoral responses (P<0.05). Comparison of G3 with G5 groups showed that IBDV had immunosuppressive effects on antibody titers against NDV and the obtained results were consistent with those reported by Cardoso, Aguiar Filho (24) who demonstrated that vaccination against IBD reduced antibody titers against ND. However, recent studies also suggested that the novel variant IBD virus could decrease the antibody titer against the NDV (25). Comparison of G4 with G1 and G3 groups demonstrated that antibody titers against NDV were also affected by aMPV.

4.3. Avian MPV

The prevalence (3 out of 20, 15%) of aMPV in broiler flocks (Figure 3) observed in this study was less than that reported in backyard birds (26). As shown in Figure 4C, antibody titer of aMPV negative flocks (G1, G2, G3, and G5) at the molecular test was also negative (titer less than 296) due to the lack of vaccination against aMPV in broiler flocks in Iran. Comparison of antibody titers of the single infected flocks (G4) with those of co-infected flocks with aMPV and IBDV (G6) indicated that IBDV had a significant (P<0.05) immunosuppressive effect on antibody titers against aMPV.

4.4. Limitation of the Study

Regarding the limitations of the present study, one can refer to the unequal number of positive flocks for IBDV, NDV, and aMPV in natural infections during a specific period of time.

5. Conclusion

Based on the obtained results, not only did subclinical IBD persist in broiler chicken flocks, but that IBDV was also involved in some co-infections with NDV and aMPV which caused immunosuppressive effects on antibody responses against these diseases. Therefore, the application of more effective vaccines against IBDV is inevitable for the reduction of immunosuppressive effects of subclinical IBD on such respiratory complex infections and improvement of immune responses to these diseases during field infections or vaccination programs, even in regions with no obvious presence of a clinical form of IBD.

Authors' Contribution

Study concept and design: M. A. and A. T.
Data collection: A. S.
Statistical analysis: A. S.
Data analysis and interpretation: M. A. and A. T.
Manuscript drafting: A. S.
Critical revision of the manuscript for important intellectual content: M. A. and A. T.
Manuscript finalization: M. A.

Ethics

All the procedures were carried out according to the standard animal experimentation protocols of the

Veterinary Ethics Committee of the Faculty of Veterinary Medicine, Urmia University, Urmia, Iran (IR-UU-AEC-3/1632/DA).

Conflict of Interest

The authors declare that they have no conflict of interest.

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Data Availability

The data presented in the current study are available upon request.

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