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Original article

CONCURRENT OCCURRENCE OF INFECTIOUS BURSAL DISEASE AND RESPIRATORY COMPLEX CAUSED BY INFECTIOUS BRONCHITIS AND AVIAN INFLUENZA (H9N2) IN BROILERS

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Summary

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Infectious bursal disease (IBD) virus is considered one of the commonest immunosuppressive diseases in chickens. The aim of this study was to investigate the concurrent occurrence of subclinical IBD in respiratory complex infections caused by avian influenza (AI, H9N2) virus and infectious bronchitis (IB) virus in broilers. During this study, 800 tissue samples of the trachea, caecal tonsil, spleen, and bursa of Fabricius and 400 blood samples were collected from 20 respiratory complex infected flocks. Detection of pathogens in the tissue samples was performed by RT-PCR for amplification of the *VP2* gene of IBD, HA region of AI, and *S1* gene of IB viruses. The amplified products were subjected to nucleotide sequence analysis. Blood samples were also tested for the detection of antibodies against IBV by using ELISA and against AIV via using the HI test. Molecular results showed that the tissue samples were positive for field isolates of subclinical IBD (45%), IB (45%), and AI-H9N2 (25%). Co-infections of IBD and IB (30%), IBD and AI (20%), and IBD, IB, and AI (5%) were also detected. Serological results indicated that subclinical IBD infected flocks had lower ($P<0.05$) antibody titres against IB and AI. In conclusion, prior exposure of broilers to IBD virus increased the incidence of respiratory complex caused by IBV and AIV in broilers, and vaccination against IBD is inevitable to reduce subclinical IBD to minimise the incidence/severity of respiratory complex diseases via improving immune responses to commonly used vaccines in broilers.

Key words: AI (H9N2), IB, IBD, respiratory complex, sequence analysis, serology

INTRODUCTION

Intervention strategies for the control of poultry diseases are mostly concentrated on addressing main causative agents, and too little attention is given to the predisposal factors (Collett & Smith, 2020). Among predisposing causes, avian immunosuppressive agents, and the IBD virus in particular, is one of the most important common predisposal wide-spread pathogens in the occurrence of respiratory complex infections in poultry (Saif & Jackwood, 2016; Umar *et al.*, 2017; Dey

et al., 2019; Eterradossi & Saif, 2020) because the IBD virus impairs the humoral, cellular, and innate immunity (Sharma *et al.*, 2000; Umar *et al.*, 2017) among which the latest acts as a primary line of defense during viral infections including AI and IB (Barjesteh *et al*., 2020). On the other hand, IBDV antigens are found in the trachea, as a main site of replication for respiratory pathogens (Singh *et al*., 2015), and extrabursal replication of IBDV may determine the cellular immunity stimulation (Rautenschlein *et al*., 2003). Therefore, the existence of IBD due to its immunosuppressive effects, even though at the subclinical form is responsible for a marked increase in occurrence and degree of severity of clinical signs of respiratory diseases (Umar *et al.*, 2017). On the other hand, avian respiratory complex infections are responsible for huge economical losses (high mortality rate, multiple treatment costs, and high carcass condemnation at slaughter) in commercial broiler chickens worldwide (Umar *et al.*, 2017). There are well-documented studies on common respiratory diseases in poultry (Almremdhy, 2014; Hassan *et al*., 2016) and among avian respiratory disease pathogens, avian influenza virus (AIV), as well as infectious bronchitis virus (IBV) are considered as the dominant respiratory pathogens whose primary replications in trachea tissue have a great impact on the occurrence of respiratory complex infections (Abo-Elkhair *et al*., 2014; Hassan *et al*., 2016). AI viruses are members of the family *Orthomyxoviridae* which replicate primarily in trachea tissue. Unlike high pathogenic AIV (HPAIV), low pathogenic AI viruses (LPAIV) require exogenous trypsin-like protease for their pathogenicity (Swayne *et al*., 2020). On the other hand, IBV is a single-stranded RNA *Gammacoronavirus* in the family *Coronaviridae* whose ciliostasis following replication in the trachea (Jackwood & de Wit, 2020) and the damaged respiratory tract of infected chickens provides vulnerable sites for other pathogenic agents, therefore IBV is a major component of mixed respiratory diseases in broilers (Hassan *et al*., 2016). Moreover, besides ciliostasis, IBV encodes a trypsin-like serine protease that enhances AIV (H9N2) pathogenicity in chickens (Hassan *et al*., 2017). This synergistic interaction not only guarantees the persistence of the pathogens but also affects the degree of severity of complex infections' outcome (Samy & Naguib, 2018). Therefore, co-infection due to AIV-H9N2 and IBV is the most predominant etiology of complex respiratory diseases in some parts of the Middle-East region (Hassan *et al*., 2016). Furthermore, co-vaccination against IBD and IB impaired immune responses against IBV (Cardoso *et al*., 2006, Umar *et al.*, 2017). Although respiratory diseases with a single avian pathogen are well documented, multifactorial co-infections under commercial conditions (natural infections) remain largely unexplored (Umar *et al.*, 2016). In addition, interactions among different avian pathogens at trachea tissue determine the outcome of co-infection, but most of the viral-viral interference studies in respiratory complex infections do not entirely reflect the field situation in which commercial broilers are routinely exposed to more than one infectious agents (Haghighat-Jahromi *et al*., 2008; Umar *et al.,* 2016, Samy & Naguib, 2018; Abdelaziz *et al*., 2019).

Therefore, this study was designed to investigate the role of subclinical IBD in the occurrence/incidence of viral respiratory complex infections due to IBV and AI in commercial broiler chickens in the

field condition. To the best of our knowledge, this study is the first comprehensive report on the occurrence of natural IBD, IB, and AI co-infections to explore the neglected parameters of these respiratory complex infections in broilers and to provide data to fill the gap between experimental and field infections. The novelty of this study may emphasise that vaccination of broilers against Gumboro disease is inevitable to prevent the occurrence of subclinical IBD to subsidise the severity of the most common important respiratory complex diseases in the poultry industry even in areas where clinical IBD is not expected.

MATERIALS AND METHODS

Ethics of experimentation

All experimental procedures were carried out according to the standard animal experimentation protocols of the Veterinary Ethics Committee of Faculty of Veterinary Medicine, Urmia University (IR-UU-AEC-1535/DA3).

Chickens

Twenty broiler chicken farms with clear clinical respiratory signs, suspected to respiratory complex infections, were selected from different regions of West Azerbaijan province of Iran from October 2018 to February 2019. Based on the isolation of IBDV, IBV, and AIV field strains, they were allocated as group 1 (farms without isolation of field strain viruses), group 2 (farms with field AI isolates), group 3 (farms with both field IBD and AI filed isolates), group 4 (farms with field IBDV, AIV, and IBV isolates), group 5 (farms with both field IBD and IB isolates), and group 6 (farms with field IB isolates).

Sampling

Eight hundred samples of trachea, spleen, caecal tonsils and bursa of Fabricius were collected from each flock individually or as an organ-specific pool (samples of 4 birds per pool) and used for RNA extraction. Furthermore, 400 blood samples were also taken from the selected broiler (at 45–50 day of age) farms for determining antibody titres against IBD, IB, and AI.

RNA extraction

RNA isolation of tissue samples was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Briefly, appropriate tissue was homogenised with 1 mL of lysis buffer and then 200 µL chloroform was added into the mixture and centrifuged at 12,000 rpm at 4 ºC for 10 min. Then phenol/chloroform (5:1) was added to the supernatant and centrifuged at 14,000 rpm at 4 ºC for 5 min. In the case of Rnase-rich tissues, treatment with phenol:chloroform was repeated three times. The supernatant was added to an equal volume of isopropanol and centrifuged at 12,000 rpm at 4 ºC for 10 min. After the washing step, the RNA pellet was completely suspended in 50 µL DW and stored at −70 °C as previously described (Saif & Jackwood, 2016).

Primers

Primers of the hypervariable (hv) region of the *VP2* gene of IBDV, *S1* gene of IBV, and *HA* gene of AIV used in this study are shown in Table 1.

Reverse transcription

Complementary DNA (cDNA) was synthesised using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Burlington, Canada) according to a previously described procedure (Najafi *et al*., 2016).

Table 1. RT-PCR primer sequences

Primers	Gene	(5'-3') Sequence	Size (bp)	References
B3F	*hvVP2*	CCCAGAGTCTACACCATA	474	Lin *et al*.,1993
B4R	*hvVP2*	TCCTGTTGCCACTCTTTC		Lin *et al*.,1993
HSAIVH9F	*H9*	CTYCACACAGARCACAATGG	330	Lee *et al*., 2001
HSAIVH9R	*H9*	GTCACACTTGTTGTTGTRTC		Lee *et al*., 2001
IBV59U391F	*S1*	GCT TTT GAG CCT AGC GTT	143	Callison *et al*., 2006
IBV59GL533	*S1*	GCC ATG TTG TCA CTG TCT ATT G		Callison *et al*., 2006

Sequence analysis

After purification of positive PCR products (Qiagen), the amplicon was sent for nucleotide sequencing in both directions (Bioneer, South Korea). Chromatograms were evaluated with CromasPro (CromasPro Version 1.5).

Phylogenetic analysis

The nucleotide sequences of the *S1* gene of IBV, *hvVP2* gene of IBDV, and *HA* (H9) gene of AI obtained in this study were subjected to BLAST, aligned, and compared with reference strains downloaded from the NCBI GenBank database. Sequence homology analysis was performed using MEGA7.0. Phylogenetic trees were constructed using MEGA7.0 with the Neighbor-Joining (NJ) algorithm (bootstrap values of 1000) with the Kimura 2 parameter model (Kumar *et al.,* 2016).

Enzyme-linked immunosorbent assay

Sera of blood samples were tested by indirect ELISA using commercial IBDV and IBV ELISA kit (Flockchek, IDEXX Laboratories, Inc., USA) to determine IBDV and IBV antibodies, according to the manufacturer's instruction. The optical density (OD) value was read at 650 nm wavelength on ELISA reader (BioTek ELX800). Results were determined by calculating the sample to positive (S/P) ratio.

Haemagglutination inhibition (HI)

Haemagglutination inhibition (HI) test was performed to determine antibody titres against the AI virus. Serum was obtained by centrifugation of samples and subjected to HI test according to the protocol of OIE (Anonymous, 2015). Briefly, two-fold serial dilutions of sera were made and 4 HA avian influenza virus subtype H9N2 with an equal volume (25 µL) of diluted sera was used in a microplate. After 45 min incubation at room temperature, 25 µL 1% chicken red blood cell was added and after 30 min incubation, the last well which had complete inhibition was considered as the antibody titer.

Statistical analysis

SPSS (version 23.0; IBM Corp, Chicago, USA) was used for analysis of the results by employing one-way ANOVA. The means of different treatments were compared with Bonferroni *post-hoc* test. Data are expressed as the mean ± standard error of mean (mean ± SEM). The differences were considered significant at $P \leq 0.05$.

RESULTS

Table 2 shows the results of detection of specific RNA for IBDV *VP2* gene, *HA* gene of AI, and *S1* gen of IBV by RT-PCR.

Table 2. Characteristics of studied IBD, IB and AI isolates

Flock No.	Age	Mortality %	Virus	RT-PCR	Virus isolate	GenBank accession No.
1	35	6.5	IBDV	+	IR/H2965-1/18	MN900865
			IBV	+	IR/Variant2/H2965-1/18	
			AIV	–	–	
2	15	12	IBDV	+	IR/H2965-2/18	MN912473
			IBV	+	IR/Mass/H2965-2/18	
			AIV	–	–	
3	22	7	IBDV	–	–	
			IBV	+	IR/Mass/H2965-3/18	
			AIV	–	–	
4	25	8	IBDV	+	IR/H2965-4/18	MN912474
			IBV	+	IR/Variant2/H2965-4/18	
			AIV	+	A/Chicken/Iran/H2965-4/2018	MN960166
6	30	12	IBDV	+	IR/H2965-6/18	MN91247
			IBV	–	–	
			AIV	+	A/Chicken/Iran/H2965-6/2018	MN960167
7	33	12	IBDV	+	IR/H2965-7/18	MN912475
			IBV	–	–	
			AIV	+	A/Chicken/Iran/H2965-7/2018	MN960168
8	40	8	IBDV	-	–	
			IBV	–	–	
			AIV	+	A/Chicken/Iran/H2965-8/2018	MN960169
9	42	5.5	IBDV	+	IR/H2965-9/18	MN912476
			IBV	+	IR/793B/H2965-9/18	
			AIV	–	–	
11	36	6	IBDV	+	IR/H2965-11/18	MN912477
			IBV	–	–	
			AIV	+	A/Chicken/Iran/H2965 11/2018	MN960170
12	35	4	IBDV	–	–	
			IBV	+	IB/793B/H2965-12/18	
			AIV	–	–	
15	29	8	IBDV	+	IR/H2965-15/18	MN912478
			IBV	+	IR/793B/H2965-15/18	
			AIV	–	–	
16	27	9	IBDV	–	–	
			IBV	–	–	
			AIV	+	A/Chicken/Iran/H296516/2018	MN960171
17	22	8	IBDV	+	IR/H2965-17/18	MN912479
			IBV	+	IR/Variant2/H296517/18	
			AIV	–	–	
20	27	6	IBDV	–	–	
			IBV	+	IR/Variant2/H296520/18	
			AIV	–	–	
5, 10, 13, 14, 18, 19	–	–	IBDV	–	–	
			IBV	–	–	
			AIV	–	–	

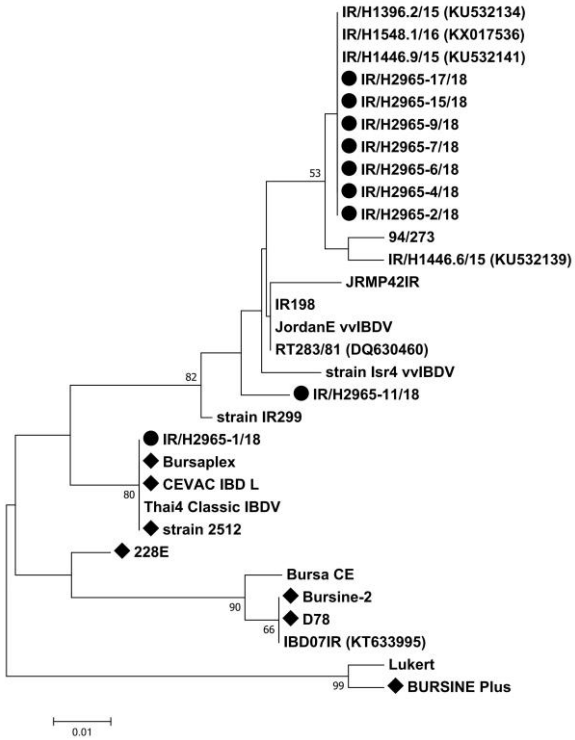


Fig. 1. Phylogenetic analysis of the *VP2* hypervariable coding sequence of 9 IBDV isolates. The viruses characterised in this report are indicated by black circle and bolded. The number of amino acids difference is indicated by the bar at the bottom of the figure. The scale bar corresponds to 0.01 substitutions per site.

Positive farms with field isolates for IBDV, IBV, and AIV were 9/20 (45%), 9/20 (45%), and 5/20 (25%) respectively. Regarding co-infection, 6/20 (30%) farms were positive for IBDV+IBV, 4/20 (20%) farms were positive for IBDV+AIV, 1/20 (5%) farm was positive for IBDV+AIV+ IBV and 6/20 (30%) farms were negative for any field isolates of the pathogens were studied. Field isolates of the IBDV, IBV, and AIV were differentiated from those of vaccinal strains by HRM and sequencing analysis.

The product of a positive sample was further processed for genetic sequencing. The nucleotide sequence of the gene was submitted to GenBank for registration. Sequences of reference strains and other related isolates were retrieved from the GenBank database. A phylogenetic tree was constructed by the neighbor-joining method with Bootstrap values, based on 1,000 replications. The phylogenetic tree was conducted with isolate to determine the genetic relationship with reported sequences with available vaccinal strains, and circulating IBDV, IBV, and AI in Iran and other countries. Phylogenetic analysis of the *VP2* hypervariable coding sequence of IBDV isolates is shown on Fig. 1, while phylogenetic analysis of the *S1* genes of IBV and of the *HA* (H9) gene of AI are presented on Fig. 2 and 3 respectively.

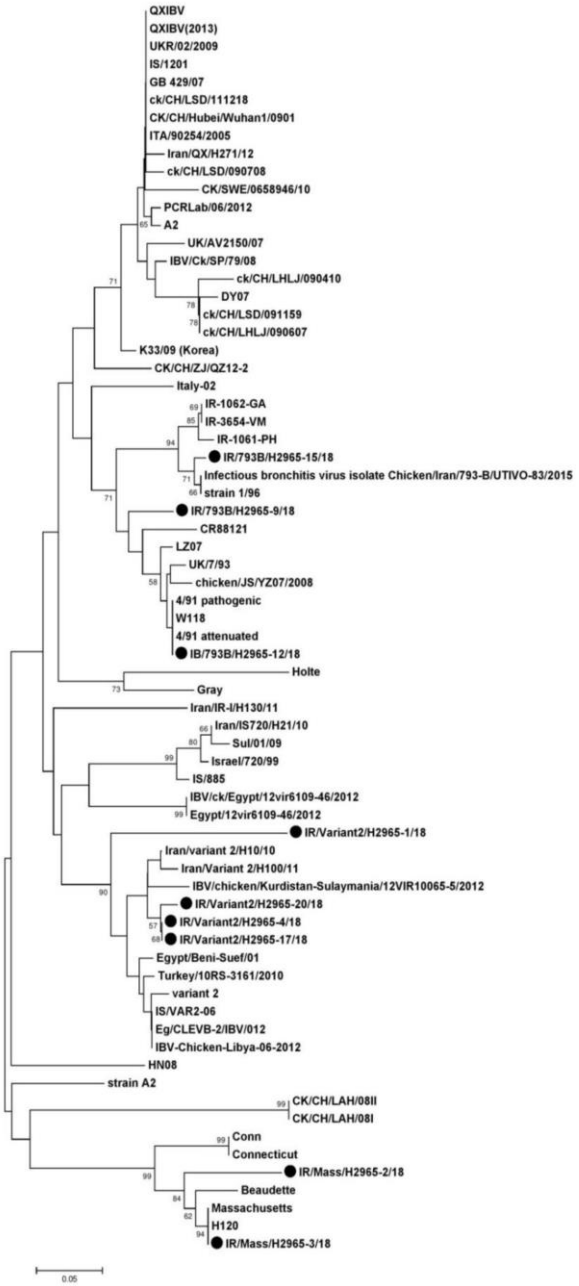


Fig. 2. Amino acid based phylogenetic relationships of *S1* genes of 9 IBVs isolated in this study. The number of amino acids difference is indicated by the bar at the bottom of the figure. The IB viruses characterised in this report are indicated by black circle and bolded. The scale bar corresponds to 0.05 substitutions per site.

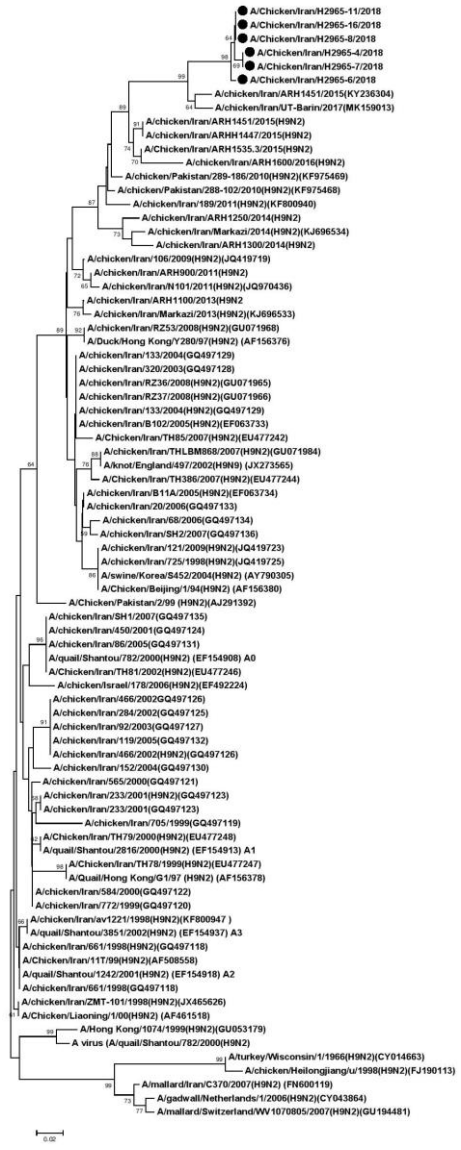
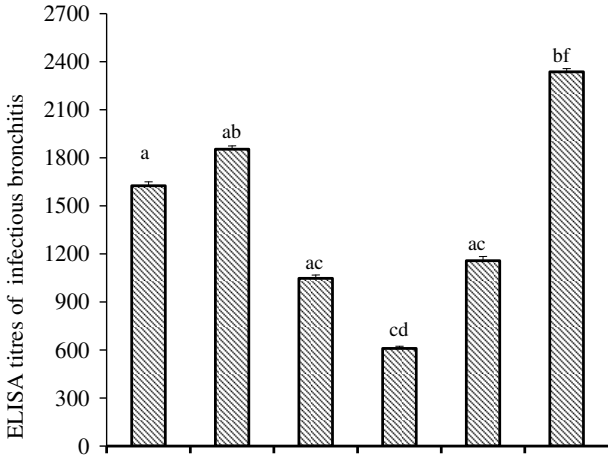


Fig. 3. Phylogenetic analysis of the HA (H9) sequence of 6 AI isolates. The viruses characterised in this report are indicated by black circle and bolded. The number of amino acids difference is indicated by the bar at the bottom of the figure. The scale bar corresponds to 0.01 substitutions per site.

ELISA antibody titres (mean ± SEM) against IB and HI antibody titres (mean ± SEM) against AI are showed on Fig. 4. ELISA antibody titres against field IB positive farms at RT-PCR for the groups 2, 3, 4, 5, and 6 were 2337, 1158, 610, 1048, and 1854 respectively, while the negative farms (group 1) had mean ELISA titre of 1626 (Fig 4A). As shown on Fig. 4B, HI antibody titres against field AI positive farms at RT-PCR were 5.4, 2.53, 4.2., 3.22, and 4.63 for groups 2, 3, 4, 5, and 6 respectively. Negative farms (group 1) had a mean titre of 4.38.

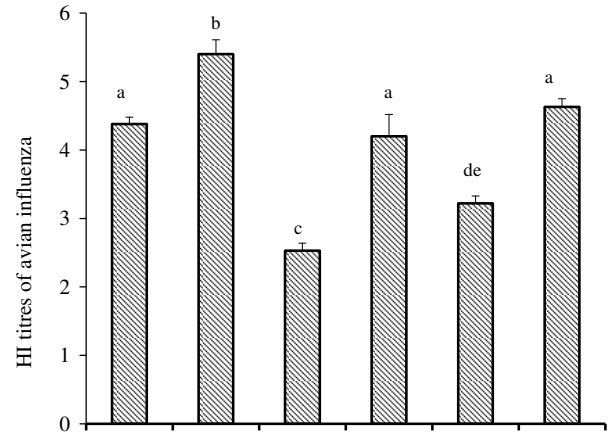
DISCUSSION

Despite routine control measures, clinical and subclinical infections with IBDV still cause immunosuppression in field conditions via damaging the respiratory ciliated epithelium and impairing both humoral (by the destruction of IgM+ cells) and cellular immune (upregulation of cytokine genes by activated IBD virus-induced bursal T cells) responses of the infected chickens (Sharma *et al*., 2000; Singh *et al*., 2015; Jackwood, 2017; Umar *et al*., 2017). According to the results of this study, field IBDV strains were isolated from 9 of 20 of the studied broiler farms (45%) indicating that although clinical forms of IBD may not be seen in some broiler farms, subclinical IBD may exist. Our results are in agreement with previous reports showing that 37 of 49 samples from different parts of Iran were positive for IBD (Peighambari & Razmyar, 2008) and that IBDV in Iran was in progressing evolution (Hosseini *et al*., 2004). Furthermore, IBD is one of the most highly widespread immunosuppressive diseases in chickens (Michel & Jackwood, 2017) and can act as a predisposal factor for the occurrence and continuous existence of



	G1	G2	G3	G4	G5	G6
IBDV	–	–	+	+	+	–
IBV	–	–	–	+	+	+
AIV	–	+	+	+	–	–

A



	G1	G2	G3	G4	G5	G6
IBDV	–	–	+	+	+	–
IBV	–	–	–	+	+	+
AIV	–	+	+	+	–	–

B

Fig. 4. Antibody titres against IB (**A**) and AI (**B**) of naturally infected groups; G1 (field IBDV, IBV, and AIV isolates negative farms), G2 (field AIV isolates positive farms), G3 (field IBD and AI isolates positive farms), G4 (field IBDV, AIV, and IBV isolates positive farms), G5 (field IBDV and IBV isolates positive farms), and G6 (field IBV isolates positive farms). Different superscript letters indicate significant differences ($P<0.05$) between the groups.

other diseases. Moreover, it has been well documented that chickens with high levels of maternal antibody to classic vaccine strains of IBDV can also be infected by vvIBDV strains and variant (subclinical IBD) (Peighambari & Razmyar, 2008). It has been reported that the majority of field infections are subclinical forms of IBD and this form becomes more prevalent in subsequent outbreaks, particularly in chickens with lacking adequate and/or uniform maternal antibody titres (Tulu, 2019; Butcher & Miles, 2020).

As shown in Table 2, the results obtained during this study showed that 6/20 (30%) of the farms had co-infections with both IBDV and IBV confirming previous reports that IBD provided an altered environment to the replicating IBV strain (Gallardo *et al*., 2012). Based on the S1 glycoprotein amino acid sequence, Iranian IBV's homology with Mass-type vaccine, and 793/B-type vaccine ranged from 54.1% to 78.5% and from 53% to 86%, respectively. The phylogenic differences between these novel IBVs and other avian coronaviruses suggest a reservoir host distinct from domestic poultry (Mardani *et al*., 2008). Serological results of this study showed that the farms with single infection by a field IBV strain positive at RT-PCR in comparison to those negative and co-infected farms had higher antibody titres against IBV (Fig. 4A) confirming the fact that single infection with IBV field strain induced a strong humoral response. Regarding to the impact of IBD on IB during a co-infection, co-infected farms with field IBDV and IBV strains (group 5) had lower antibody titres against IBV when compared with group 6, indicating that IBDV field strain had immunosuppressive effects on antibody titre against the IB infection as shown in Fig. 4A. Furthermore, the co-infected farm with field IBD, IB and AI strains (group 4) had the lowest antibody titres in comparison to groups 5 and 6, indicating that AI had also effects on IB. The results obtained in this study are in agreement with previous (mostly experimental) reports (Lindahl, 2004; Cardoso *et al*., 2006; Hassan *et al*., 2017; Umar *et al*., 2017), and these findings may explain the failure of the routine vaccinations in the protection of broiler chickens against several distinct circulating IBV genotypes (Mass, 793/B, IS720, Variant 2, QX, IR-I, and IR-II) in Iranian poultry farms (Hosseini *et al*., 2015).

The results of this study also showed that 4/20 (20%) of the farms had concurrent infection with field IBDV and AIV (H9N2) (Table 2). The results (Fig. 4B) indicated that antibody titres of co-infected flocks with field IBD and AI strains were lower than those with a single infection with field AIV strains. Our results are in agreement with the previous reports that IBD immunosuppression affects AI infection outcomes and the IBDV-immunosuppressed chickens produced significantly lower antibody titre against AIV (Motamed *et al*., 2013, Spackman *et al*., 2017; Hashemzade *et al*., 2019; Ranjbar *et al*., 2019).

As shown in Table 2, co-infection of IBD, IB, and AI was observed at one farm (5%). Although there are some reports regarding natural occurrence of IB+AI co-infection (Seifi *et al*., 2010; Boroomand *et al*., 2018; Haji-Abdolvahab, 2019), unfortunately we did not come across documented reports on co-infection with IBD, IB, and AI, therefore comparison on the incidence rate of the co-infection with these three diseases could not be made. As shown on Fig. 4A, the co-infected farm with field IBDV, IBV, and AIV isolates had antibody titres lower than those at

farms with a single infection of field IB isolates indicating that IBD had a suppressive effect on the IB. Therefore this study could be considered as the first report for the simultaneous natural occurrence of these three avian diseases. Higher antibody titres on the farms infected with IBDV, IBV, and AIV in comparison to antibody titres of farms infected with IBDV and AIV could be attributed to the trypsin-like serine protease encoded by IBV enhancing the pathogenicity of AIV (H9N2) as well as inducing a severe clinical outcome, high mortality and high shedding of AIV (H9N2) (Cardoso *et al*., 2006; Seifi *et al*., 2010; Hassan *et al*., 2017; Belkasmi *et al*., 2020).

In conclusion, subclinical IBD was highly prevalent in broiler farms; prior exposure of broilers to IBD virus increased the incidence of respiratory complexes caused by IBV, as well as AIV subtype H9N2 in broilers. Vaccination against IBD is inevitable to reduce its subclinical form to minimise the incidence of respiratory complex diseases via improving immune responses to commonly used vaccines in broilers.

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