Original Article

The role of the major viral pathogens in a respiratory disease outbreak of broiler flocks in Eastern Iran

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10.22099/IJVR.2023.45051.6632

(Received 25 Oct 2022; revised version 6 May 2023; accepted 3 Jun 2023)

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Abstract

Background: Infectious bronchitis virus (IBV), Newcastle disease virus (NDV), and avian influenza virus (AIV) H9N2 are major viral pathogens in broiler respiratory disease. **Aims:** Following a respiratory disease outbreak and economic losses in eastern Iran 2020-2021, we investigated the role of major viral pathogens and the implemented vaccination programs. **Methods:** Thirty-six respiratory disease affected broiler flocks in South Khorasan province were sampled, molecularly tested, and coinfections were investigated. The vaccination programs were obtained and the detected IBV were genotyped. **Results:** IBV, virulent NDV, and AIV H9N2 were detected in twenty-five, seven, and seven flocks, respectively. IBV+AIV, IBV+NDV, and NDV+AIV coinfections were respectively detected in six, five, and one flocks. Most IBV infected flocks (84%) had been immunized with a live IBV-Mass vaccine. All NDV infected flocks and 14.2% of AIV infected flocks had been vaccinated. IBV genotyping showed a high prevalence of variant 2 (83.3%), followed by Mass-type (12.5%), and Q1-type (4.2%). Variant 2 IB viruses were widely distributed in the province and half of them were mostly similar to the ones that had been detected in northern neighboring province, Khorasan Razavi. **Conclusion:** Single infection with variant 2 IBV was a major cause of the respiratory disease outbreak in which use of the Mass vaccine was probably not effective. The high coverage and multiple doses of vaccination against Newcastle disease possibly had reduced the prevalence of NDV. Considering the regional origin of IBV strains, strong biosecurity measures should be implemented and vaccination programs using appropriate vaccine strains should be used.

Key words: Avian influenza virus, GI-16, GI-23, Newcastle disease virus, Vaccine strain

Introduction

Respiratory disease can significantly impact the broiler industry through reduced production and economic losses (Pantin-Jackwood and Spackman, 2020). Coinfection with multiple pathogens is expected in the field and causes multifactorial respiratory disease (Patnayak *et al*., 2004). Multiple infections usually exacerbate the clinical signs; however, infection with one pathogen may also prevent infection with a secondary agent (Pantin-Jackwood and Spackman, 2020). In commercial chicken flocks, coinfection with several viruses is prevalent due to the high density and large flock size (Roussan *et al*., 2008; Benachour *et al*., 2015). Infectious bronchitis virus (IBV), Newcastle disease virus (NDV), and avian influenza virus (AIV) are major viral pathogens to cause respiratory disease in commercial chicken flocks, either alone or through coinfection (Roussan *et al*., 2008; Sid *et al*., 2015).

Infectious bronchitis (IB) is a highly contagious respiratory disease with global distribution and causes significant economic losses in the commercial poultry industry (Pantin-Jackwood and Spackman, 2020). The causative agent, IBV, was first detected in North Dakota, USA, in the 1930s (Schalk and Hawn, 1931). IBV primarily replicates in the respiratory tract of chickens and causes respiratory disease. However, some strains, by infecting kidney and ovarian epithelial cells, cause egg production loss, nephritis, and death (Schalk and Hawn, 1931). Spike (S) protein, the major antigen of IBV with approximately 1145 amino acids, breaks into two subunits S1 and S2 following translation (Cavanagh, 1983). Although S2 is a conserved protein, the S1 protein sequence is variable and determines the virus serotype, virulence, and tissue tropism (Cavanagh, 1983). The S1 gene contains three hypervariable regions (HVR) (amino acids 38-67, 91-141, and 274-387), and the third HVR is usually used for virus genotyping (Cavanagh *et al*.,

1988). IBV is classified into six genotypes, including 32 distinct viral lineages and some inter-lineage recombinants (Valastro *et al*., 2016). Genotype I (GI) contains 27 lineages, and the remaining five genotypes include one lineage. Lineages like GI-23 (Variant 2), GI-1 (Mass type), GI-19 (LX4 or QX), GI-13 (793B type), GI-21 (Italy02), and GI-16 (Q1) have been detected in several continents, while others are limited to specific regions of the world (Valastro *et al*., 2016). Commercial chicken flocks have been vaccinated against IB for years in Iran; however, IBV infection still causes respiratory disease and economic losses in Iranian commercial broiler flocks (Homayounimehr *et al*., 2016; Hamadan *et al*., 2017).

Newcastle disease (ND), caused by NDV or avian paramyxovirus 1, is endemic in Iran (Molouki *et al*., 2019, 2021; Allahyari *et al*., 2022). Since the first report of ND in Indonesia in 1926, the disease has caused significant economic losses to the world's poultry industry (Suarez *et al*., 2020). NDV pathotypes include asymptomatic enteric (avirulent), lentogenic, mesogenic (moderately virulent), and velogenic (very virulent) (Suarez *et al*., 2020). Unlike IBV, all known NDV strains have one serotype and are classified into two major classes based on phylogenetic analysis using the fusion (*F*) gene (Dimitrov *et al*., 2019).

Class I NDV demonstrates low genetic diversity and are mostly non-pathogenic, while class II NDV demonstrates high genetic diversity (18 genotypes) and different pathotypes (Dimitrov *et al*., 2019). Genotype VII of class II viruses are common in Middle Eastern countries (Dimitrov *et al*., 2019). Despite the occasional detection of exotic NDV genotypes such as XIII (Hejazi *et al*., 2022), recent studies in Iran have shown that velogenic strains under genotype VII.1.1 are prevalent in commercial chicken flocks (Molouki *et al*., 2019, 2021; Allahyari *et al*., 2022).

AIV H9N2, a primary pathogen in multifactorial broiler respiratory disease, is endemic in Middle Eastern countries, including Iran (Nili and Asasi, 2003; Naeem *et al*., 2007; Pantin-Jackwood and Spackman, 2020). AIV belongs to the family Orthomyxoviridae and the genus Alphainfluenzavirus. Based on the surface glycoproteins of the virus, including hemagglutinin (HA) and neuraminidase (NA), AIV has different subtypes (ICTV, 2018). AIV is categorized into high pathogenicity avian influenza (HPAI) and low pathogenicity avian influenza (LPAI), considering the pathogenicity in chicken (Lupiani and Reddy, 2009). H9N2 viruses belong to the category of LPAI; however, in the complex with other viruses and bacteria, they can lead to significant mortality (Pantin-Jackwood and Spackman, 2020).

South Khorasan province is located in eastern Iran and borders with Afghanistan. The province borders four Iranian provinces, including Khorasan Razavi in the north, Semnan, Isfahan, Yazd, and Kerman in the west, and Sistan Baluchestan in the south. South Khorasan province is ranked 14th among Iranian provinces by commercial broiler chicken population, with more than 750 flocks. Following an outbreak of respiratory disease in commercial broiler flocks of the province, we implemented the most comprehensive study in eastern Iran investigating the role of IBV, NDV, and AIV H9N2 in the respiratory disease outbreak. Considering the importance of vaccination in the prevention of viral diseases, the effect of vaccination programs was investigated in the studied flocks, considering the prevalence of viruses. The thorough information obtained from this study is essential to plan effective respiratory disease control programs.

Materials and Methods

Study population, sampling, and vaccination programs

During a respiratory disease outbreak in commercial broiler flocks of South Khorasan province, eastern Iran, thirty-six flocks with respiratory disease and mortality were sampled during autumn and winter 2020-2021. Farm veterinarians diagnosed the respiratory disease by observing the clinical signs (swollen sinuses, nasal discharge, wheezing, sneezing, and gasping) and postmortem lesions (tracheal hyperemia and/or obstruction, lung lesions, hypoxic discoloration of carcass). The trachea, lung, and kidney tissues of five newly dead birds from each flock were sampled aseptically. The samples were sent to the laboratory on the ice pack and were stored at -70°C before nucleic acid isolation.

The flocks located in seven districts of South Khorasan province, including Khusf (three flocks), Ghayen (eleven flocks), Birjand (nine flocks), Darmiyan (five flocks), Boshrouyeh (one flock), Ferdows (one flock), and Sarbisheh (six flocks) (Fig. 1). The size of the flocks ranged from 6000 to 58760 chickens. Mortality rate varied from 0.52 to 100% (average 24.49%).

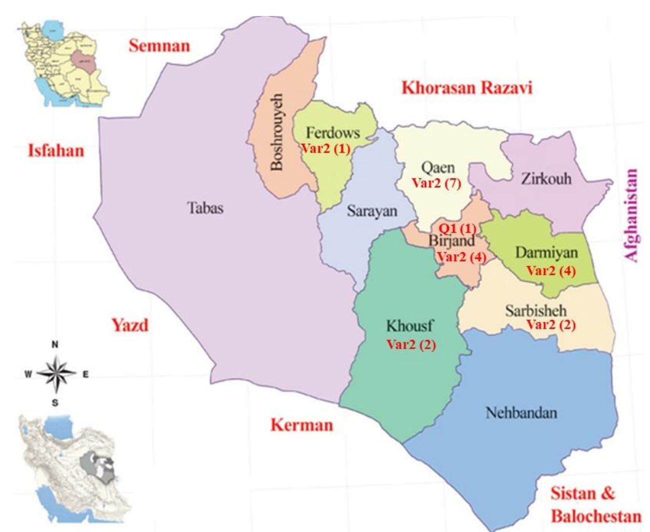

Fig. 1: Geographical map of the distribution of IBV genotypes in South Khorasan province. The number of positive flocks corresponding to each genotype is enclosed in parentheses. The map is retrieved from the governorship of South Khorasan province website: https://www.sko.ir/page-SkoEnglish/en/0/form/Cl468/link

Twenty-nine flocks (80.6%) had been vaccinated against IB with different doses (Table 1). Live H120 vaccine was the only vaccine strain used in the studied flocks.

Thirty-two flocks (88.9%) had received vaccination against ND with different doses (Table 1). The ND vaccines included B1, ND.TR.IR, PHY.LMV.42, V4, clone, LaSota, and NDV6/10. The vaccines were live except V4 vaccine.

In the case of AI immunization, twelve flocks (33.3%) had been vaccinated once with an inactivated H9N2 vaccine (Table 1).

Preparation of samples and viral RNA isolation

The pooled samples for each flock were prepared by homogenizing 70% suspension of the trachea, lung, and kidney tissues in PBS buffer and centrifuging at 3,000 g for 15 min at 4°C. 300 µL of supernatant of each pooled sample was used for viral RNA isolation with SinaPure Viral kit (Sinaclon, Tehran, Iran) according to the manufacturer's instruction. The quality and quantity of the extracted RNA were evaluated using a NanoDrop 2000c spectrophotometer (NanoDrop Products, Wilmington, USA). The extracted RNA was used directly to make cDNA, and the remaining RNA was stored at -70°C.

cDNA synthesis

In reverse transcription (RT) reaction, the ExcelRT™ reverse transcription enzyme (SMOBIO, Hsinchu, Taiwan) was used according to the manufacturer's instruction. For IBV detection, SX2 primer specific for the *S1* gene was used for RT reaction (Table 2) (Worthington *et al*., 2008). For NDV detection, primer A complementary to the *F* gene was employed for the RT reaction (Table 2) (Kant *et al*., 1997). To detect AIV H9N2, H9-151f primer specific to the *H9* gene was used for cDNA synthesis (Table 2) (Lee *et al*., 2001).

RT reactions were performed separately for each pathogen in a total volume of 10 µL. Total RNA and primer concentrations for each RT reaction were 100 ng and 10 pmol, respectively.

RT-PCR, nested and seminested PCR

Nested PCR for IBV detection was performed using XCE1, SX2, SX3, and SX4 primers (Table 2) (Cavanagh *et al*., 1999; Worthington *et al*., 2008). These primers are common to most known strains of IBV and amplify HVR3 of the *S1* gene. The H120 vaccine virus was used as a positive control of molecular assays. PCR was performed using Taq polymerase 2X Master Mix (Ampliqon, Odense, Denmark), 15 pmol of each primer, including XCE1 and SX2, and 1 µL of the cDNA in a total volume of 20 µL. Nested PCR was performed using 1 µL of a one to ten dilution of the PCR product as a template, 15 pmol of each primer including SX3 and SX4; and Taq polymerase 2X Master Mix in a total volume of 20 µL. The amplification temperature profile comprised a melting step of 94°C for 3 min; 35 cycles of 3-step including 94°C for 25 s, 50°C for 30 s, and 72°C for 30 s; and a final extension step of 72°C for 10 min. For IBV positive samples, nested PCR produces a DNA band of about 390 bp on the agarose gel. The size of PCR products was confirmed by agarose gel electrophoresis at a concentration of 1.5%.

For molecular detection of NDV, seminested PCR employing primers A, B, and C was used (Table 2) (Kant *et al*., 1997). The sequences of these primers are complementary to the conserved regions of the fusion gene. A and B primers bind to the genomic sequence of non-virulent and virulent NDV; however, primer C is specific to virulent NDV. PCR was done employing Taq polymerase 2X Master Mix, 15 pmol of each primer, including A and B, and 1 µL of the cDNA in a total of 20 µL. Seminested PCR was performed using 1 µL of a one to ten dilution of the PCR product as a template, 15 pmol

Table 1: Vaccination coverage against IBV, NDV, and AI H9 based on the number of doses used in 36 studied flocks

Vaccine type	Total number of vaccinations among flocks					
	None	Once	Twice	Three times	Four times	Five times
IBV	7 (19.4%)	7 (19.4%)	14 (38.9%)	7 (19.4%)	1 (2.8%)	-
NDV	4 (11.1%)	5 (13.9%)	6 (16.7%)	9 (25%)	6 (16.7%)	6 (16.7%)
AI H9	24 (66.7%)	12 (33.3%)	-	-	-	-

Table 2: Sequence and position of the primers used in RT-PCR, nested and seminested PCR for detection of viruses

Virus (gene)	Oligonucleotide	Sequence (5´-3´)	Position in S1 sequence	Reference
IBV (S1)	XCE1 +	CACTGGTAATTTTTCAGATGG	728 to 749	Cavanagh *et al*. (1999)
	SX2 -	TCCACCTCTATAAACACCYTT	1148 to 1168	Worthington *et al*. (2008)
	SX3 +	TAATACTGGYAATTTTTCAGA	705 to 725	Worthington *et al*. (2008)
	SX4 -	AATACAGATTGCTTACAACCACC	1075 to 1097	Worthington *et al*. (2008)
NDV (F)	A	TTGATGGCAGGCCTCTTGC	141 to 159	Kant *et al*. (1997)
	B	GGAGGATGTTGGCAGCATT	503 to 185	Kant *et al*. (1997)
	C	AGCGTYTCTGTCTCCT	395 to 380	Kant *et al*. (1997)
AIV (H9)	H9-151f	CTYCACACAGARCACAATGG	151 to 170	Lee *et al*. (2001)
	H9-638r	GTCACACTTGTTGTTGTRTC	619 to 638	Lee *et al*. (2001)

of each primer, including A and C, and Taq polymerase 2X Master Mix in a total volume of 20 μL. The amplification temperature profile was the same as the one for IBV detection. A and C primers amplify a 254 bp fragment, which is detectable by 1.5% agarose gel electrophoresis.

RT-PCR using H9-151f and H9-638r primers specific to the *H9* gene was employed for AIV detection (Table 2) (Lee *et al.*, 2001). PCR was done employing Taq polymerase 2X Master Mix, 10 pmol of each primer, including H9-151f and H9-638r, and 1 μL of the cDNA in a total volume of 25 μL. The amplification temperature profile was the same as the one for IBV detection. In the case of AIV H9N2 presence in the sample, the PCR product of 488 bp was detectable by 1.5% agarose gel electrophoresis.

Phylogenetic analysis

Since nested PCR alone cannot distinguish between pathogenic and vaccine strains of IBV, nested PCR products were sequenced by Pishgam Biotech Company (Tehran, Iran) using specific primers. The chromatograms were examined by BioEdit software version 7.5.2, and the results were edited if needed. For phylogenetic analysis, *S1* gene HVR3 sequences of twenty-seven IBV strains with defined genotypes and some Iranian IBV strains were obtained from GenBank (Valastro *et al.*, 2016). Sequences were aligned using ClustalW implemented in BioEdit software version 7.5.2 (Hall *et al.*, 2011). MEGA-X software was used for the best model selection and tree construction (Kumar *et al.*, 2018). The maximum likelihood (ML) statistical method, Hasegawa-Kishino-Yano model, and G substitution model were used. The levels of support for relationships were estimated through 1000 bootstrap replicates.

Statistical analysis

SPSS version 16 (SPSS, Chicago, IL, USA) was used for data analysis. Flock size and mortality values were respectively transformed to log10 and square-root data to obtain a normal distribution. ANOVA was used to investigate the relationship between each virus infection rate and flock size. The Chi-square test was used to compare two groups with mortality rates ≤30 and >30% in terms of infection with the studied viruses. When more than 20% of the expected frequencies were less than 5, Fisher's exact test was used. Kruskal-Wallis was employed to investigate the relationship between each virus infection rate and the number of vaccinations. We used ANOVA to test if there was a significant difference between the numbers of vaccination and mortality. P-values <0.05 were considered statistically significant.

Results

Clinical and postmortem findings

Besides detecting the mentioned clinical respiratory signs and postmortem lesions in all flocks, we detected renal inflation and hyperemia in nine IBV positive flocks (36%) and proventriculitis in three NDV positive flocks (43%). Neurologic signs, including ataxia, lameness, and inability to move, were seen just in one flock that was IBV, NDV, and AIV H9N2 positive.

Molecular detection of IBV, NDV, and AIV H9N2 and vaccination programs in infected flocks

Among the respiratory disease affecting commercial broiler flocks, IBV, NDV, and AIV H9N2 were detected with different prevalence rates (Table 3). We found that IBV was highly prevalent in the studied flocks compared to NDV and AIV H9N2.

IBV was detected in twenty-five flocks (69.44%, 95% CI: 54.4-84.5%) located in Birjand (seven flocks (28%)), Ghayen (seven flocks (28%)), Darmiyan (five flocks (20%)), Sarbisheh (three flocks (12%)), Khusf (two flocks (8%)), and Ferdows (one flock (4%)). Among the flocks infected with IBV, 16% had not been vaccinated, 28% had received one dose of vaccine, and 56% were vaccinated against IB twice or more (Tables 3 and 4).

There was no significant association between IBV infection rate and flock size (F (1, 34) = 1.08, P=0.3). Investigation of the relationship between IBV infection rate and mortality showed that the prevalence of IBV infection in flocks with a mortality rate of >30% was significantly higher than in flocks with a mortality rate of ≤30 (χ^2=5.36, df=1, N=36, and P=0.02) (Table 4). There was no significant association between IBV infection and the number of vaccinations, χ^2 (3, N=36) = 4.43, P=0.21.

Virulent NDV was detected in seven flocks (19.44%, 95% CI: 6.5-32.4%) located in districts of Khusf (two flocks (29%)), Ghayen (two flocks (29%)), Darmiyan (one flock (14%)), Ferdows (one flock (14%)), and Birjand (one flock (14%)). All NDV infected flocks were vaccinated against ND. 28.6% of flocks had received one dose of vaccine and 71.4% had been vaccinated with two doses or more against the disease (Tables 3 and 4).

There was no significant association between NDV infection rate and flock size (F (1, 34) = 0.73, P=0.39). There was no relationship between NDV infection rate and mortality rates ≤30 and >30% (Fisher's exact test, P=0.43). We found no association between NDV infection and the number of vaccinations, χ^2 (4, N=36) = 2.87, P=0.57.

AIV H9N2 was detected in seven flocks (19.44%, 95% CI: 6.5-32.4%) located in districts of Birjand (three flocks (43%)), Sarbisheh (one flock (14%)), Khusf (one flock (14%)), Ghayen (one flock (14%)), and Ferdows (one flock (14%)). Of the seven AIV H9N2 infected flocks, one flock (14.2%) was vaccinated against AI (Tables 3 and 4). Eleven (38%) out of the twenty-nine AIV H9N2 negative flocks were vaccinated against the AI.

There was no significant association between the AIV H9 infection rate and flock size (F (1, 34) = 2.67, P=0.11). There was no difference among the AIV H9 infected and noninfected flocks in the case of vaccination status, χ^2 (1, N=36) = 1.05, P=0.3. There was no relationship between the AIV H9 infection rate and

mortality rates ≤30 and >30% (Fisher's exact test, P=0.56).

We found no significant difference among the numbers of vaccination and mortality regarding IBV (F (3, 32) = 0.02, P=0.99), NDV (F (4, 31) = 0.33, P=0.85), and AIV H9 (F (1, 34) = 0.4, P=0.52) infections.

Single and multiple infections

Among IBV, NDV, and AIV H9N2 positive flocks, the single infection percentage was 60, 28.6, and 14.3%, respectively. IBV + AIV H9N2, IBV + NDV, and NDV + AIV H9N2 coinfections were respectively detected in six (16.6%), five (13.9%), and one (2.8%) flocks.

The prevalence of IBV genotypes

Phylogenetic analysis showed that out of twenty-five genotyped IBV, twenty (83.3%), three (12.5%), and one (4.2%) IBV strains were classified in GI-23 (variant 2), GI-1 (Mass-type), and GI-16 (Q1-type) lineages, respectively (Figs. 1 and 2).

Variant 2 IBV strains were detected in six districts of Ghayen (seven flocks), Birjand (four flocks), Darmiyan (four flocks), Khusf (two flocks), Sarbisheh (two flocks), and Ferdows (one flock). Except for Ferdows, variant 2 infected flocks are in adjacent districts of Ghayen, Birjand, Darmiyan, Khusf, and Sarbisheh (Fig. 1).

Table 3: Characteristics of broiler flocks with respiratory disease and the results of molecular tests for IBV, NDV, and AIV H9N2

Flock	Flock size	Sampling age (day)	Mortality (%)	Vaccine type (day(s) of application)			IBV PCR (genotype)	NDV PCR	AI H9 PCR
1	24000	41	12.5	H120 (6,12)	ND (6,19,40)		+ (Var2)	-	+
2	43200	30	48.6	H120 (1,17)	ND (1,8,25)	H9N2 (8)	+ (Var2)	-	-
3	28000	50	1.8	H120 (2,10)	ND (2,6)	H9N2 (6)	+ (Var2)	-	-
4	13500	24	37.2				+ (nd[5])	-	+
5	42880	44	23.3	H120 (1,7)	ND (1,7,12,23)		-	+	-
6	20400	51	22.1	H120 (1,14)	ND (1,7,14,21)	H9N2 (7)	-	-	-
7	30000	27	26.7	H120 (1)	ND (1,7,11)	H9N2 (7)	+ (Var2)	-	+
8	31200	28	73.1		ND (29)		+ (Var2)	+	-
9	32000	31	6.2	H120 (5,6,10)	ND (6,19)		+ (Var2)	-	-
10	24000	50	8.6	H120 (1,8)	ND (1)		-	+	-
11	18900	15	2.1		ND (13)		-	-	-
12	21600	56	4.6	H120 (5)	ND (5)		-	-	-
13	11000	32	50	H120 (6)	ND (6,19,26)		+ (Var2)	-	-
14	21000	25	55.4	H120 (3,9,16)	ND (16,22,41)		+ (Var2)	+	-
15	50000	50	12	H120 (1,9)	ND (1,9,23)		+ (Var2)	-	-
16	6000	31	57.7	H120 (6)	ND (6,12,32)		+ (Var2)	-	-
17	58760	50	16.5	H120 (2,7,10)	ND (10,20,30)		-	-	-
18	11300	31	11.5	H120 (11)	ND (11,20,30)		+ (Q1)	-	+
19	22000	25	19.8				-	-	-
20	12000	30	58.3	H120 (7)	ND (7,13,27)	H9N2 (7)	+ (Var2)	-	-
21	25000	27	100		ND (21,23)		+ (Var2)	-	-
22	18000	27	100	H120 (1,7)	ND (1,7,14,27)	H9N2 (7)	+ (Var2)	-	-
23	16500	31	7	H120 (7)	ND (7,15,27)		+ (Var2)	-	+
24	32000	28	18.7		ND (13, 20, 25)		-	-	-
25	14400	25	11.7	H120 (1,7)	ND (1,7,13,24)	H9N2 (7)	+ (Mass)	-	-
26	33000	50	18.2	H120 (2,10)	ND (2,8,15,25)	H9N2 (8)	+ (Var2)	-	-
27	15600	27	100	H120 (6,11)	ND (6,20,25)	H9N2 (6)	+ (Mass)	-	-
28	14500	40	50.13	H120 (5,10)			-	-	-
29	28800	43	11.5	H120 (10,12,18)	ND (12,47)		-	-	-
30	42000	28	74.6	H120 (5)			+ (Mass)	-	-
31	12000	28	100	H120 (8,13,16)	ND (8,16)		-	-	+
32	14000	39	36.5		ND (7,12,23,26)		+ (Var2)	+	+
33	11000	43	74.6	H120 (5,13)	ND (5,13,21,28)	H9N2 (5)	+ (Var2)	-	-
34	27000	29	41.9	H120 (8,26)	ND (8,26)		+ (Var2)	+	-
35	17600	40	63.1	H120 (1)	ND (1)		+ (Var2)	-	-
36	22000	45	19.1	H120 (5)	ND (5,9,17)	H9N2 (9)	+ (Var2)	+	-

Table 4: Flock size, mortality, and vaccination coverage in flocks with positive and negative molecular tests

Virus	Flock infection		Flock size (mean±SD)	Mortality %		Vaccination coverage
				≤30	>30	
IBV	Pos.	25	22772 ± 11321	27.8[a]	41.7[a]	84%
	Neg.	11	26894 ± 13574	25	5.6	73%
NDV	Pos.	7	26011 ± 9147	8.3	11.1	100%
	Neg.	29	23554 ± 12695	44.4	36.1	86.2%
AIV H9	Pos.	7	17329 ± 7026	11.1	8.3	14.3%
	Neg.	29	25650 ± 12481	41.7	38.9	34.5%

[a] The values are significantly different, P<0.05

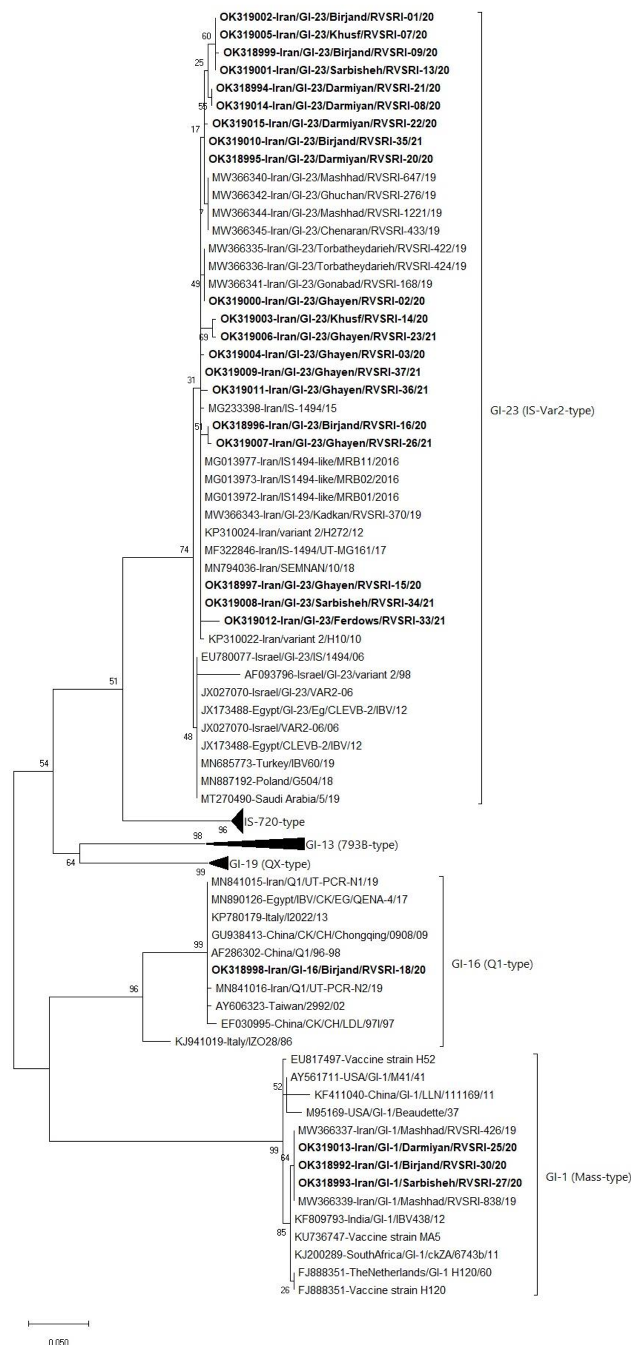

Fig. 2: Maximum likelihood phylogenetic tree of *S1* gene partial nucleotide sequences. The evolutionary tree is drawn based on 1000 bootstrap replicates. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 103 nucleotide sequences. There were a total of 314 positions in the final dataset. IBV strains detected in this study were bolded. Evolutionary analyses were conducted in MEGA X

15% of variant 2 infected flocks had not been vaccinated against IB. 25% of flocks had not received one vaccine dose and 60% had been vaccinated with two or three doses against IB (Table 3). Three Mass IBV strains were detected in Birjand, Darmian, and Sarbisheh. These flocks had been vaccinated against IB (Table 3). Among IBV infected flocks, we detected a Q1-type IBV infected flock in Birjand. The flock had been vaccinated once against IB (Table 3).

As seen in the constructed phylogenetic tree, variant 2 IBV strains detected in Darmiyan (OK318995, OK319015, OK319014, and OK318994), Birjand (OK319002, OK318999, and OK319010), Khusf (OK319005), and Sarbisheh (OK319001) placed at the same node as variant 2 IBV strains (MW366340, MW366342, MW366344, and MW366345) detected in the three adjacent cities of Mashhad, Ghuchan, and Chenaran, located in Khorasan Razavi province (Fig. 2).

Nucleotide sequence identity between the variant 2 viruses detected in South Khorasan province ranged from 98.41 to 100%. Nucleotide sequence identity between the variant 2 viruses detected in Khorasan Razavi province and South Khorasan province ranged from 98.41 to 99.68%.

A variant 2 IBV detected in Ghayen (OK319000) was placed in the same branch with three viruses detected in Torbatheydarieh and Gonabad cities in Khorasan Razavi province in 2019 (MW366335, MW366336, and MW366341). Gonabad is located in the south of Khorasan Razavi province and is adjacent to Torbatheydarieh in the north and Ghayen in the south.

S1 partial gene sequences of positive samples were submitted to GenBank, and accession numbers OK318992 to OK319015 were assigned to the viruses.

Discussion

IBV, NDV, and AIV H9N2 are significant viral pathogens causing respiratory disease in Iranian commercial chicken flocks (Nili and Asasi, 2003; Homayounimehr *et al*., 2016; Hamadan *et al*., 2017; Molouki *et al*., 2019, 2021; Allahyari *et al*., 2022). During 2020-2021, a respiratory disease outbreak negatively affected broiler flocks of South Khorasan province and led to significant economic losses. Accordingly, following the most comprehensive study in east Iran, we investigated the presence of the major viral pathogens as single or multiple infections in the respiratory disease affected flocks.

We found that most studied flocks (69.44%) were IBV infected. Phylogenetic analysis showed that 20 of 25 IBV positive flocks had variant 2 IBV infection, a pathogenic genotype involved in renal and respiratory pathologies (Meir *et al*., 2004). Variant 2 IBV detected in ten flocks were similar to the viruses detected in Khorasan Razavi province in 2019 (Tabatabaeizadeh *et al*., 2021). The other variant 2 viruses had the most similarities with IB viruses reported from other regions of Iran (Fig. 2). The similarity of variant 2 viruses between the two neighboring provinces indicates a regional virus spread and a gap in implementing biosecurity programs.

Q1 IBV was detected in one of the studied flocks. Before the present study, Q1 IBV infection was reported in northern Iran in 2019 (Ghalyanchilangeroudi *et al*., 2020). Analysis of the Q1 IBV showed a 100% similarity

to the previously detected Q1 genotype in Iran (Ghalyanchilangeroudi *et al*., 2020). Q1 IBV is a pathogenic genotype that causes respiratory disease, proventriculitis, severe egg drop, and nephropathogenic disease (Yu *et al*., 2001; De Wit *et al*., 2012; Toffan *et al*., 2013). Considering that this is the second case of detecting Q1 genotype in Iran after three years, we speculate that the predominance of variant 2 in the country has negatively affected the spread of Q1 genotype. However, the detection of a Q1 virus in this study indicates that this genotype remained undetected in the country.

Among the studied viruses, IBV infection was significantly higher in flocks, with a mortality rate of more than 30%. This finding, along with the high prevalence of the pathogenic strains of IBV, can indicate the important role of IBV infection in the outbreak of respiratory disease in eastern Iran. However, it should be considered that the variable rate of mortality observed in the studied flocks may be the result of a complication with several different factors such as viruses, bacteria, mycoplasma, inappropriate management, and environmental conditions, as well as immunosuppressive agents. These conditions that often occur in commercial settings (Pantin-Jackwood and Spackman, 2020).

Variable rates of IBV prevalence were reported in different years and regions of Iran. In a study of IBV outbreak in South Khorasan province and Sistan Baluchestan in 2015, a lower overall prevalence (37.5%) was reported, and variant 2 (25%) was the most prevalent genotype (Ghalyanchi-Langeroudi *et al*., 2015).

Similar to the present study, a high overall prevalence of IBV (84.7%) and variant 2 genotype (85%) was reported in nine Iranian provinces (Ghalyanchilangeroudi *et al*., 2019). In another study in Khorasan Razavi province in 2019, the overall prevalence of IBV (73.3%) and variant 2 genotype (72.7%) were similar to the present study (Tabatabaeizadeh *et al*., 2021).

Some Iranian studies reported a lower overall prevalence of IBV (30-52%); however, variant 2 IBV was the most prevalent (22-36%) detected genotype (Hamadan *et al*., 2017; Gholami *et al*., 2018; Rahimi *et al*., 2018). In a study of 40 broiler flocks with respiratory disease in Fars and Chaharmahal Bakhtiari provinces in 2016, 30% of the flocks were IBV positive, and variant 2 prevalence was 22.5%. Similar to the present study, most IBV positive flocks (58%) had been immunized against IBV using the H120 vaccine, and the age of the affected flocks was 20 to 40 days (Rahimi *et al*., 2018).

In studies performed in Iran in the early 2010s, variant 2 IBV was less prevalent than other genotypes (Hosseini *et al*., 2015; Homayounimehr *et al*., 2016). However, a recent study also found a remarkably lower prevalence of variant 2 (5%) compared to other genotypes (Ghorbiani *et al*., 2020).

Among the flocks infected with IBV, we found a high coverage (84%) of IB vaccination, so that 56% of flocks had received at least two vaccine doses. However, IBV infection was not significantly related to vaccination coverage and the number of vaccine doses. This finding may indicate that the use of the Mass vaccine strain may not be effective against infection and disease with variant 2 IBV. Some experimental studies have also shown similar results (Awad *et al*., 2015; De Wit *et al*., 2017).

We found that 19.44% of the flocks had virulent NDV infection. NDV prevalence in the outbreak was low, compared to other studies in Iran. Studies of Iranian broiler flocks showed a virulent NDV infection rate of 25-35% (Alian Samakkhah *et al*., 2019; Molouki *et al*., 2019; Maki *et al*., 2022).

NDV-infected flocks in the present study were immunized against ND with a variable number of vaccine doses and timing. The employed ND vaccine strains provide protection and prevent mortality. However, the shedding of the field virus may not be prevented (Dimitrov *et al*., 2017). Considering that out of seven flocks infected with NDV, five were coinfected with variant 2 IBV, the detection of virulent NDV in these flocks may have been due to infection and not the disease.

The use of different vaccine strains, high vaccination coverage, and multiple-dose vaccination may be considered as one of the factors for the low prevalence of ND in South Khorasan province. However, in some NDV infected flocks, the single dose (flock 10) and late (flocks 8 and 14) vaccination show that the vaccination programs against ND need to be improved.

Considering the prevalence of genotype VII NDV in Iran (Molouki *et al*., 2019, 2021; Allahyari *et al*., 2022) and due to the antigenic differences among Newcastle disease vaccine strains, employing selected vaccine strains such as the V4 compared to the Lasota vaccine can be more effective in preventing Newcastle disease caused by genotype VII viruses (Tabatabaeizadeh, 2021; Elbestawy *et al*., 2023). Also, using virulent field strains of NDV as a vaccine strain can enhance vaccine protection and reduce virus shedding compared to classical vaccine strains (Miller *et al*., 2007, 2009; Roohani *et al*., 2015; Fawzy *et al*., 2020; Khabiri *et al*., 2023).

We found that 19.44% of the flocks were infected with AIV H9N2. Most of the previous studies in Iran were serologic, and compared to the present study, a much higher prevalence was reported. Seroprevalence of AIV H9N2 in domestic chicken in northern Iranian provinces and broiler flocks in 13 provinces of Iran were 56 and 77%, respectively (Fallah Mehrabadi *et al*., 2020; Ebrahimi *et al*., 2021). Compared to molecular studies, serology does not show a current infection and may remain positive following an early infection. Accordingly, a higher prevalence may be expectable in serology compared to molecular testing. In a molecular study of 100 broiler flocks in the neighboring country Afghanistan during 2016-2017, a higher prevalence (40%) was found (Hosseini *et al*., 2017).

We found the lowest vaccination coverage (33.3%) against AIV H9N2, and among seven AI H9N2 infected flocks, one flock was vaccinated against the virus. Accordingly, even with the low prevalence of the virus

in South Khorasan province, increasing vaccination coverage is required to reduce the current prevalence rate and prevent any possible AIV outbreak.

Researchers investigated the concomitant role of IBV, NDV, and AIV infections in multifactorial broiler respiratory disease. In a study on AIV H9N2, NDV, and IBV, 233 broiler flocks in 31 Iranian provinces were tested from 2015 to 2016 (Haji-Abdolvahab *et al*., 2019). The prevalence of IBV was similar to the present study (72.9%); however, multiple infections (47.2%) were more common than single infections (25.7%). The most common genotypes among IBV infected flocks were 793/B (31.6%) and variant 2 (28.3%), and a higher prevalence of virulent NDV (39%) and AIV H9N2 (31.3%) was reported by Haji-Abdolvahab *et al*. (2019). Of the seven flocks sampled in South Khorasan province, 3, 2, and 0 were infected with IBV, NDV, and AIV H9N2, respectively (Haji-Abdolvahab *et al*., 2019). Regarding the difference between the prevalence of viruses in South Khorasan province compared to the present study, the importance of flock numbers and their geographical distribution in provincial studies should be considered to obtain reliable results.

In a study in Saudi Arabia for molecular detection of viral pathogens in 10 respiratory disease affected broiler flocks, IBV, NDV, and AI H9N2 prevalence were 40, 40, and 30%, respectively (Al-Ali *et al*., 2018). Similar to the present study, a study of 89 broiler and layer flocks with respiratory disease in Pakistan showed the prevalence of AI H9N2 (16.8%) and NDV (17.9%); however, the IBV prevalence (4.4%) was low (Umar *et al*., 2019).

In conclusion, we found that the pathogenic strains of IBV were highly prevalent in South Khorasan province. On the other hand, the prevalence of NDV and AIV H9N2 was low compared to other regions of Iran. Accordingly, IBV was the major viral pathogen involved in the broiler respiratory disease outbreak in South Khorasan province. This study shows that high coverage and multiple doses of H120 strain vaccination were ineffective in preventing IB caused by variant 2 IBV. On the other hand, the vaccination programs for ND should be improved, especially in terms of timing, and in the case of influenza, the vaccination coverage should be increased. At the same time, in future studies, taking into account the vaccination programs implemented in a group of healthy flocks, it is possible to comment with more certainty in this regard. Due to the proven efficacy of using a combination of Mass and 793B vaccines, respectively, at the ages of 1 and 14 days against variant 2 and Q1 viruses (Awad *et al*., 2015; De Wit *et al*., 2017), we recommend an appropriate IB vaccination program. Importantly, regional circulation of variant 2 IBV emphasizes the importance of strong biosecurity measures to prevent such outbreaks.

Acknowledgements

The authors are grateful to the Razi Vaccine and Serum Research Institute (RVSRI) and the South Khorasan Veterinary Service Head Office for providing funds and facilities to conduct this study.

Conflict of interest

The authors declare no financial or conflict of interest regarding this study that could inappropriately influence the work.

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