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Natural co-infection caused by avian influenza H9 subtype and infectious bronchitis viruses in broiler chicken farms

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ABSTRACT

Although H9N2 AIV (Avian Influenza Virus) is pathotyped as a low pathogenic avian influenza virus, our extensive field experiences over the last decade show serious disease and high mortality in broiler chicken associated with this subtype in many regions of Iran. One of the possible explanations for such high mortality and great economic losses could be mixed infection with other respiratory pathogens such as infectious bronchitis virus (IBV). This investigation was conducted to study the natural co-infections of H9N2 AIV and infectious bronchitis viruses in broiler chicken flocks in Fars province, Iran. Tracheal samples were taken from chickens in 30 closely monitored broiler flocks suffering from respiratory disease with mortality higher than normal range. RT-PCR was performed using primers in order to detect the M protein and the H9 gene of avian influenza A. The multiplex nested RT-PCR was also performed to detect Massachusetts, 793B (4/91) and D274 serotypes of IBV in the samples. In this study, AIV and IBV were detected in 16 (53/3%) and 12 (40%) out of 30 flocks, respectively. The tracheal samples of 11 out of 12 flocks were positive for 4/91 strain of IBV and one for Massachusetts serotype. A mixed infection of AIV (H9 subtype) and IBV (4/91 serotype) was observed in four flocks with severe lesions and 20-30% mortality. The results of this study indicate the high occurrence of natural co-infection of AIV and IBV in Iranian broiler chicken farms showing respiratory signs and they show that the mortality rate in co-infected flocks was significantly higher than others ($P<0.05$).

Key words: avian influenza, infectious bronchitis, co-infection

Introduction

Avian influenza (AI) is a highly contagious disease caused by type A influenza viruses, a member of the family *Orthomyxoviridae* (SWAYNE and SUAREZ, 2000). Based on surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA), these viruses

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are divided into subtypes (WEBSTER et al., 1992). At present, 16 HA (H1-H16) and 9 NA (N1-N9) subtypes have been recognized (FOUCHIER et al., 2005). Avian influenza viruses can be divided into two distinct groups on the basis of their ability to cause diseases in poultry (CAPUA and ALEXANDER, 2004). Highly pathogenic avian influenza viruses (HPAIV) may cause up to 100% mortality. At present, these viruses have been restricted to subtypes H5 and H7, although not all viruses of these subtypes cause HPAI. On the other hand, low pathogenic avian influenza viruses (LPAIV) cause mild to moderate infections in various domestic and wild bird species (MO et al., 1997; ALEXANDER, 2000). Among the LPAI, the H9N2 subtype has been most studied, mainly due to its pandemic potential, its successful transmission to humans and also the fact that it contains internal genes needed for the human transmission of the H5N1 subtype, causing severe infections in domestic poultry in the last decade (GUO, 2002; TWEED et al., 2004). Prior to 1990, H9N2 influenza viruses were mainly reported from avian species in North America (GUO et al., 2000) and were reported only in ducks in southeastern China (SHORTRIDGE, 1992), while these viruses have become widespread in domestic chickens in Asian and Middle Eastern countries over the last decade (GUAN et al., 1999).

Since 1998, H9N2 AI outbreaks occurred in chicken farms in Iran and rapidly spread throughout the country. Although laboratory examinations in SPF chickens show that the H9N2 avian influenza virus is low pathogenic (VASFI MARANDI and BOZORGMEHRIFARD, 1999; SWAYNE and HALVORSON, 2003), there were several reports about outbreaks of H9N2 infection with high mortality (NAEEM et al., 1999; ALEXANDER, 2000; BANO et al., 2003). Our frequent field observations showed that severe respiratory infections in affected chicken flocks resulted in mortality up to 65% in broiler chicken farms and the most prominent lesions in affected dead birds were respiratory airway hyperemia and severe exudation, which lead to tubular cast formation in the tracheal bifurcation, extending to the lower bronchi (NILI and ASASI, 2002, and 2003). Co-infection with other respiratory pathogens may complicate the respiratory disease syndrome during outbreaks of non-highly pathogenic avian influenza viruses and cause severe disease and high mortality.

Some strains of infectious bronchitis viruses (Shiraz3, 4/91 and H120) were isolated from several broiler flocks during the course of the H9N2 outbreak in Iran (NOURI et al., 2003; SEYFI-ABAD SHAPOURI et al., 2002 and 2004; HAQSHENAS et al., 2005). In spite of regular vaccinations with Massachusetts (Mass.) strains, IBV still has severe and diverse effects on the poultry industry in the country, causing mortality and decreasing the quantity and quality of egg production. This investigation was conducted to detect AIV (H9 subtype) and IBV viruses and the possibility of their co-infection in broiler chicken flocks in Fars province, Iran.

Materials and methods

Thirty broiler chicken flocks with severe respiratory symptoms were closely monitored for clinical signs, gross lesions and mortality in Fars province, Iran. The age of the affected flocks, vaccination programs, environmental rearing conditions, clinical signs, gross lesions and mortality during the course of the disease were recorded. Ten dead birds selected randomly from the flocks were subjected to necropsy and their lesions were recorded. The specimens were submitted to the laboratory in cold condition. RNAs were extracted from the mucosal epithelium of the tracheal specimens as described previously. Briefly, 1 mL of RNX solution (a commercial RNA extraction kit, CinnaGen, Iran) was added to 100 mg of each sample of tissue scraping, then 200 µL of chloroform was add to the mixture. After centrifugation of the samples at 12000 rpm for 15 min., the aqueous phases were transferred to another tube. RNA was precipitated with the addition of an equal volume of isopropanol. After washing the sediment with 75% ethanol, it was eluted in 50µL of distilled water and stored at -70 °C until used.

The S1 gene of IBV was detected using a nested multiplex RT-PCR. The published primers (XCE1 and XCE2) were used in reverse transcription and in the first step of RT-PCR (ADZHAR et al., 1997). In brief after a 5 min incubation of 20 pmol of the primers with 5 µL of extracted RNA, the mixture were transferred to the 0.2 mL tube containing a lyophilized master mix (AccuPower® RT PreMix kit, BioNeer, South Korea) for reverse transcription. The reaction was performed at 42 °C for 1 h with the total volume of 20 µL. The same primers were used in the first step PCR using lyophilized master mix (AccuPower PCR PreMix kit, BioNeer, South Korea) containing Taq DNA polymerase 1 µL, each dNTP(dATP, dCTP, dGTP, dTTP) 250 µM, Tris-HCl (Ph:9.0) 10 mM, KCl 40 mM and $MgCl_2$ 1.5 mM. The program in Ependorf thermal cycler was 94 °C for 5 min and 35 cycles including: 94 °C for 30 sec. 50 °C for 30 sec. 72 °C for 35 sec. and a post polymerization step at 72 °C for 2 min. The second step of PCR was performed using 1 µL of the first PCR product and 10 pmol of the forward primers MCE1, DCE1 and BCE1 specific for detection of the serotypes Massachusetts, D274 and 4/91, respectively (ADZHAR et al., 1997). A common reverse primer, XCE3, was used for all of these serotypes. PCR products including 295, 217 and 154 bp were tested for the serotype Massachusetts, D274 and 4/91, respectively. The thermal cycler parameters were the same as described above, only the post-polymerization step was 4 min. The products were analyzed in a 1% agarose gel containing ethidium bromide, using an ultraviolet transilluminator.

For detection of the AIV genome, the cDNA was synthesized for the M protein gene using AccuPower® RT PreMix kit (BioNeer, South Korea) according to the manufacturer's instructions. Five µL of RNA template and 20 pmols of each primers (Random hexamer and AIMCD; Table 1) were used for cDNA preparation. Then the PCR was performed using primers, CN1 and CN2 (Table 1), that were designed in our laboratory. PCR was

performed to amplify a 450 bp fragment of the M protein gene of the influenza A virus, using the AccuPower PCR PreMix kit (BioNeer, South Korea) in a 20 µL reaction mixture containing 5 µL cDNA and 10 pmols of each primer. Amplification was performed using the thermal cycler for 35 cycles of denaturation at 94 °C for 35 sec., annealing at 54.9 °C for 45 sec. and polymerization at 72 °C for 45 sec. The post polymerization was at 72 °C for 5 min.

Table 1. The sequences and positions of the oligonucleotide primers used in RT-PCR

Oligonucleotide	Sequence	Gene	Position in the sequence
Infectious bronchitis			
XCE2-	CTCTATAAACACCCTTACA	S1	1168 to 1193
XCE1+	CACTGGTAATTTTTCAGATGG	S1	728 to 749
XCE3-	CAGATTGCTTACAACCACC	S1	1093 to 1111
BCE1+	AGTAGTTTTGTGTATAAACCA	S1	958 to 978
DCE1+	ATACAATTATATCAAACCAGC	S1	895 to 915
MCE1+	AATACTACTTTTACGTTACAC	S1	817 to 837
Avian influenza			
CN1	GGGAAGAACACAGATCTTGAGG	M	100 to 121
CN2	TGC TGG CTA GCA CCA TTC TC	M	531 to 550
H9 Forward primer	CTYCACACAGARCACAATGG	H9	151 to 171
H9 Reverse primer	GTCACACTTGTTGTTGTRTC	H9	618 to 638
AIMCD	TCTAACCGAGGTCGAAACGTA		

For identification of H9 serotype in AI positive samples for M protein the cDNA was synthesized using AccuPower® RT PreMix kit (BioNeer, South Korea) according to the manufacturer's instructions. The primers were specific for the H9 protein gene as described by LEE et al. (2001) (Table 1). Five µL of RNA template and 20 picomols of each H9 specific primers were used for cDNA preparation. PCR was performed to amplify 488 bp fragment of the H9 protein gene of the influenza A virus, using the AccuPower PCR PreMix kit (BioNeer, South Korea) in a 20 µL reaction mixture containing 5 µL cDNA and 10 pmols of each primer. The reaction mixture was subjected to 35 cycles of 94 °C, 53 °C and 72 °C for 35 sec, 45 sec and 45 sec, respectively, followed by final extension at 72 °C for 5 min. The PCR products were separated in 1% agarose gel and visualized under ultraviolet light.

Statistical analysis. The data of mortality were analyzed using Chi-Square exact test (by GraphPad inStat 3.06 software) to find out the significance of differences between flocks infected with H9N2 and IBV and also dual infection.

Results

The findings of the field study and the laboratory tests on 30 broiler chicken flocks with respiratory signs and relatively high mortality in Fars province (Iran) are summarized in Table 2. The course of the uncomplicated disease in most flocks was 7-8 days. The most significant clinical signs observed in depressed chicks were sneezing, coughing and tracheal rales. Some clinical signs such as ruffled feathers, swelling of the periorbital tissues and sinuses, conjunctivitis and nasal and ocular discharge were also observed. Gasping with head raised and neck extended was the first obvious sign in severe cases. Infected flocks showed feed intake reduction during the clinical sign exhibition, much more in the early phase. No association could be seen between clinical signs and RT-PCR results, except decreased feed consumption in co-infection of AI and IB. Although the mortality rates were variable in different flocks, as shown in Table 2, death occurred from 2 days after the appearance of clinical signs then continued as a spiking curve pattern, reaching a total mortality rate of up to 30% during the course of the disease. The noticeable gross lesions in the necropsy finding were congestion of the tracheal mucosa and the existence of excessive exudates with a tendency to form tubular casts in the tracheal bifurcation extending to the lower bronchi (Fig. 1 a,b). The airways were heavily occluded with casts of caseous exudate in some cases leading to asphyxia. No airsacculitis, pericarditis, perihepatitis and other lesions due to bacterial contamination on the viscera were observed in dead birds in the early phase of the disease as well as in antibiotic treated flocks during the course of the illness. Fewer flocks showed complications due to poor ventilation and management. The later ones experienced prolonged mortality as a result of air sac disease and right ventricular failure.

As shown in figures 2, 3 and 4, amplification of expected DNA bands from the positive control confirmed that the reaction was performed correctly. Sixteen out of 30 flocks (53.3%) were positive for the H9 protein gene of AIV. The S1 protein gene of infectious bronchitis virus was detected in 12 out of 30 flocks (40%) either vaccinated with the H120 strain of IBV or not vaccinated. The samples of 11 flocks out of 12 positive flocks for the S1 protein gene of IB were 4/91 strain and one was the Mass serotype. In four cases mixed infection of IBV (4/91 strain) and AIV (H9 subtype) were observed. The D274 serotype of IBV was not detected in this study.

Table 2. The summarized history of the selected flocks suffering from respiratory disease

No. of selected flocks	Flocks size	Age (days)	Vaccination[a]		Clinical signs[b]	Gross lesions[b]	Mortality[c] (%)	AI RT-PCR	IB RT-PCR d
			IB	AI					
1	11000	28	+	+	1+	1+	5%	-	+
2	12000	28	-	+	1+	1+	5%	-	+
3	16000	22	+	+	1+	1+	4%	-	-
4	10000	25	+	+	2+	2+	7%	+	-
5	7000	37	+	-	3+	3+	25%	+	+
6	11200	52	+	-	3+	3+	15%	-	+
7	7000	28	+	-	3+	3+	19%	+	-
8	8000	38	+	-	3+	3+	30%	+	+
9	30000	40	-	+	3+	3+	20%	+	-
10	20000	30	+	+	3+	3+	20%	-	+
11	15000	37	+	+	3+	3+	15%	+	-
12	6000	25	+	+	3+	3+	15%	+	-
13	26000	21	-	+	1+	1+	5%	-	-
14	8000	25	+	+	2+	2+	8%	+	-
15	12000	18	-	+	3+	3+	20%	+	+
16	10000	21	+	+	1+	1+	4%	-	-
17	9000	22	+	-	2+	2+	9%	+	-
18	12000	35	+	+	3+	3+	15%	+	-
19	8000	21	-	+	3+	3+	22%	+	+
20	6000	37	-	+	1+	1+	5%	-	-
21	15000	28	+	+	3+	3+	18%	+	-
22	7000	28	+	+	1+	1+	4%	-	-
23	5500	24	-	+	3+	3+	12%	+	-
24	5000	32	+	+	2+	2+	8%	-	+
25	10000	26	+	+	2+	2+	10%	-	+
26	7000	35	-	+	3+	3+	11%	+	-
27	7800	28	+	+	1+	1+	3%	-	-
28	7000	25	-	+	2+	2+	8%	+	-
29	9000	21	-	+	2+	2+	8%	-	+
30	5000	28	+	+	2+	2+	8%	-	+

[a] Vaccine used against infectious bronchitis was a live attenuated vaccine of H120 strain administered orally at the first week of life, AI vaccines were inactivated ones. Vaccination against ND and IBD were based on official order and local veterinarian administration. No other vaccines used. [b] Clinical signs such as; depression, ruffled feathers, coughing and tracheal rales, sneezing, swelling of the periorbital tissues and sinuses, conjunctivitis, nasal and ocular discharge; and gross lesions including; congestion of the tracheal mucosa and existence of exudates and tubular casts in the tracheal bifurcation and in bronchi were scored as: 1+ : mild, 2+: moderate, and 3+: sever. [c] Mortality was accounted for during the course of the disease (7-8 days). [d] All IB strains detected were 4/91 except flock No.30.

The mortality rate analysis among AIV (H9 subtype), IBV (4/91 strain) and dual infected flocks showed significant differences so that dual infected flocks had a higher mortality rate ($P<0.05$). Six flocks with mild respiratory clinical signs and low mortality were negative in RT-PCR tests for AIV and IBV.

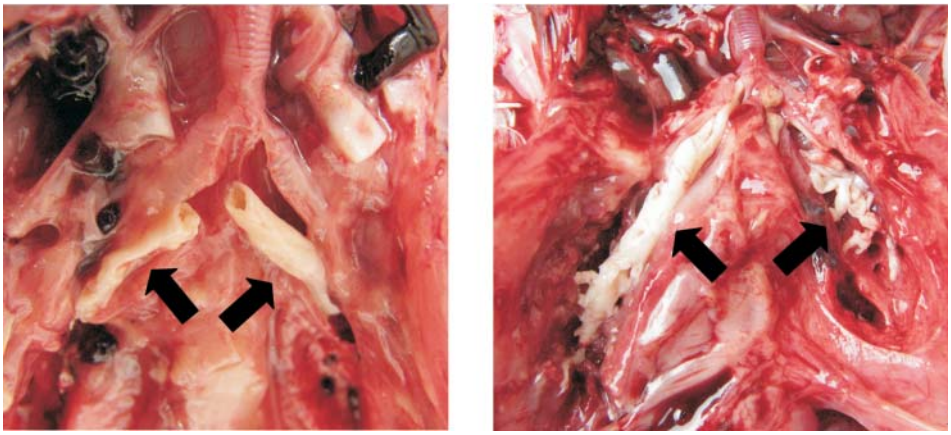

Fig. 1. (a and b). Heavily fibrinonecrotic caseous cast formation (arrow heads) in secondary and lower bronchi of birds which died from natural H9N2 AI co-infected with IB (4/91 strain)

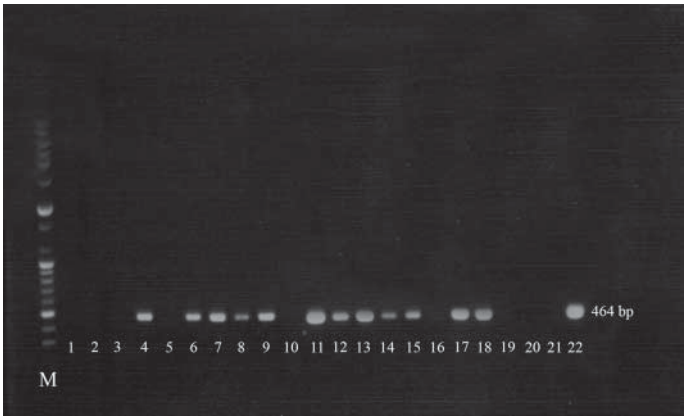

Fig. 2. IBV RT-PCR products, M: DNA marker (100 bp), L22: positive control, L1: blank, L4, L6-9, L11-15, L17 and L18: positive samples, L2-3, L10, L16, and L19-21: negative samples

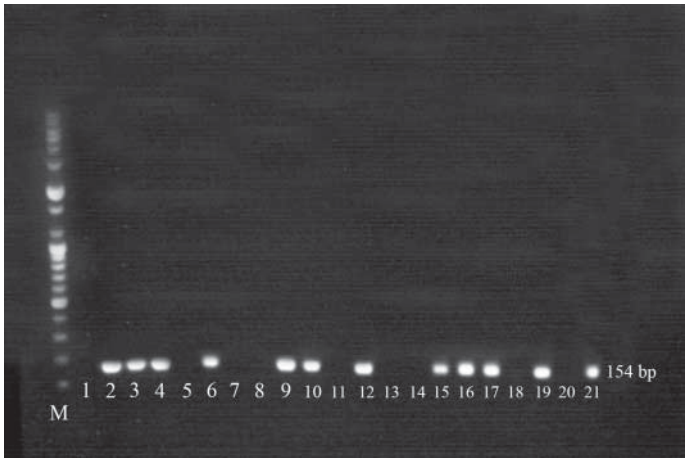

Fig. 3. IBV nested multiplex RT-PCR products, M: DNA marker (100 bp), L21: positive control, L1: blank, L2-4, L6, L9-10, L12, L15-17 and L19: positive samples, L5, L7-8, L11, L13-14, L18 and L20: negative samples

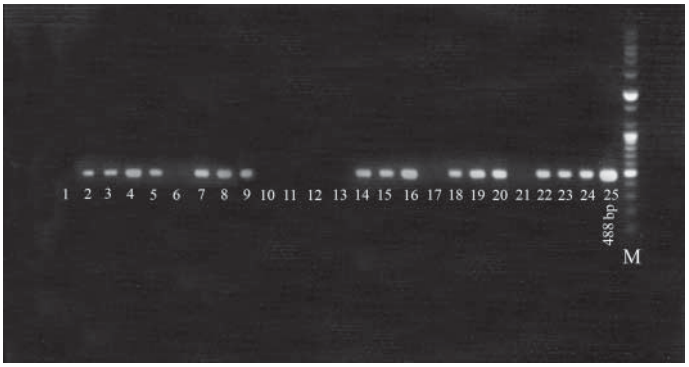

Fig. 4. AIV PCR products (H9 protein gene), M: DNA marker (100bp), L25: positive control, L1: blank, L2-5, L7-9, L14-16, L118-20 and L22-24: positive samples, L6, L10-13, L17, and L21: negative samples

Discussion

Over the last 10 years, influenza viruses of the H9N2 subtype have been isolated from outbreaks in poultry in various countries such as Germany, Italy, Ireland, Saudi Arabia, Iran, Pakistan, China, Hong Kong, South Africa, and the United States (BANKS et al., 2000; ALEXANDER, 2000 and 2007; CAPUA and ALEXANDER, 2004). Laboratory examination of SPF chicken showed that H9N2 avian influenza virus is low pathogenic,

but in the last decade Asian and Middle Eastern countries have faced frequent outbreaks of H9N2 infection with high mortality (NAEEM et al., 1999; GUO et al., 2000; BANO et al., 2003). It was reported that outbreaks of H9N2 influenza viruses in Iranian broiler chicken farms caused a 20-65% mortality rate, from 1998 to 2001 (NILI and ASASI, 2002 and 2003), however, it is well documented that low pathogenic avian influenza viruses, such as the H9N2 subtype in domestic poultry manifest mild clinical signs and respiratory diseases with low mortality, not exceeding 5% (SWAYNE and HALVORSON, 2003). It is proposed that concurrent infection may play a key role in exacerbating mortality in chicken infected with mild AIVs. To consider the factors which may increase the mortality of broiler chicken infected with AIV H9N2 subtype, the present study was designed to detect IB viruses circulating in flocks suffering from respiratory symptoms leading to high mortality in Fars province, Iran. The RT-PCR methods used in this study for the detection of IBV and AIV have been shown to be able to detect these agents correctly (ADZHAR et al., 1997; CAVANAGH et al., 1999; LEE et al., 2001). In this study AIV (H9 subtype) was detected in 53.3% of the flock samples taken. The clinical signs and gross lesions seen in the affected flocks in this study were comparable with what was described previously as the natural and experimental non-highly pathogenic AI H9N2 subtype (VASFI MARANDI and BOZORGMEHRIFARD, 1999; GUO et al., 2000; NILI and ASASI, 2002 and 2003; BANO et al., 2003), severe disease conditions were observed in 4 out of 30 flocks with co-infection of AI (H9 subtype) and IB (4/91 strain) simultaneously. In spite of the vaccination program, the mortality in these simultaneous co-infected flocks was 20-30%. The results of this study indicate that combination infections with AIV and IBV cause more severe respiratory signs and mortality than either agent alone ($P<0.05$) and that the pathogenicity of AIV H9N2 is increased when combined with IBV. Some strains of IBV (Shiraz3, 4/91 and H120) were isolated from several broiler flocks during the course of the H9N2 outbreak in Iran (NOURI et al., 2003; SEYFI-ABAD SHAPOURI et al., 2004; HAQSHENAS et al., 2005). The results of this study also showed the high occurrence of 4/91 serotype of IBV in Fars province (Iran). This strain of IBV was first isolated in 1985 in France (CAVANAGH et al., 1998). Then it spread to many countries in Europe, Japan, Saudi Arabia, Thailand and Mexico (COOK et al., 1996; CAVANAGH et al., 1999). Despite the regular vaccinations on chicken farms, mostly with Mass. strains, IB still has a severe adverse effect on the poultry industry in Iran. Cross-protection between different serotypes of IBV is variable, hence vaccination failure may be due to low homology (26%) between the 4/91 strain (793/B) and Mass-type vaccines such as the H120 strain (CAVANAGH et al., 1997; CAVANAGH, 2003; PARSON et al., 1992; AKBARI AZAD et al., 2007). It has been demonstrated that the infectious bronchitis (IB) live vaccine, H120 strain, also exacerbates the manifestation of experimental H9N2 AIV infection in broiler chicken (HAGHIGHAT-JAHROMI et al., 2008). IBVs could provoke ciliostasis in the host's

ciliated airways (COOK et al., 1976); and may therefore facilitate the opportunity for other related pathogens to induce their pathogenicity. On the other hand, it is well known that trypsin-like proteases are necessary for the cleavage activation of the HA and thus play a key role in viral pathogenicity (BOSCH et al., 1979; KLENK and GARTEN, 1994). IB co-infection may have provided the enzymes and enhanced H9N2 pathogenicity in the affected flocks in this study. It has been reported that a trypsin-like serine protease domain is encoded by coronavirus IBV (LIU et al., 1995; NG and LIU, 2000). The presence of similar enzymes in the field situation could possibly increase the pathogenicity of H9N2 AIV.

Surprisingly, the signs and lesions seen in chickens where only IBV was detected were the same as the only H9N2 AIV positive flocks. The trachea is a primary target for IBV isolation and is, therefore, the preferred sampling site in the early days of post infection (DE WIT, 2000), however, there is a possibility that the virus could have disappeared from tracheal mucosa during the sampling process in some AIV only infected cases.

Other co-infections of the MPAIV infection with bacterial and viral agents have also been described. KISHIDA et al. (2004), demonstrated that the co-infection of H9N2 with *Staphylococcus aureus* and *Haemophilus paragallinarum* activated the HA of the influenza virus and allowed multiple cycles of virus replication. BANO et al. (2003) also showed that *Escherichia coli* have a significant role in viral shedding and the virulence of H9N2 pathogenicity. However, in the present study none of the cases subjected to necropsy showed any lesions indicating concurrent bacterial infection. Our frequent field experience indicates that high mortality could be seen in farms infected with the H9N2 subtype of AI, with poor hygienic condition. Six flocks with RT-PCR negative for both IB and AI showed mild respiratory clinical signs and lesions and experienced less than 5% mortality. Further research needs to be done to elucidate the complexity of high mortality in broiler chicken infected with H9N2 AIV.

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SAŽETAK

Premda podtip H9N2 virusa influence ptica (VIP) po patotipu pripada slabo patogenom virusu influence ptica, terenska iskustva posljednjih desetak godina pokazala su da je pojava teške bolesti i velikog pomora tovnih pilića u mnogim dijelovima Irana vezana uz taj podtip. Jedno od mogućih objašnjenja za tako visok pomor i velike gospodarske gubitke mogla bi biti mješovita zaraza s ostalim uzročnicima dišnih bolesti kao što je virus zaraznog bronhitisa (VZB). Ovo istraživanje poduzeto je s ciljem proučavanja prirodne mješovite zaraze virusom influence ptica H9N2 i virusom zaraznog bronhitisa u tovnih pilića u pokrajini Fars u Iranu. Uzorci obriska dušnika bili su uzeti od pilića na 30 promatranih jata tovnih pilića u kojih je bila ustanovljena dišna bolest s pomorom većim od uobičajenog. Rabljen je RT-PCR s početnicama specifičnima za dokazivanje proteina M i gena za hemaglutinin H9 virusa influence ptica A. Višestruka ugniježđena RT-PCR rabljena je za dokaz serotipova Massachusetts, 793B (4/91) i D274 u pretraživanim uzorcima. VIP je bio dokazan u 16 (53/3%), a VZB u 12 (40%) od 30 pretraženih jata. Uzorci dušnika uzeti iz 11 od 12 jata bili su pozitivni za soj 4/91 VZB i jedan za serotip Massachusetts. Mješovita infekcija virusom influence ptica podtipom H9 i virusom zaraznog bronhitisa (serotip 4/91) bila je promatrana na četirima jatima ptica s teškim poremećajima i 20-30%-tnim pomorom. Rezultati ovog istraživanja govore o pojavi prirodne koinfekcije virusom influence ptica i virusom zaraznog bronhitisa u iranskih tovnih pilića koji su pokazivali dišne znakove te da je pomor u jatima s mješovitom zarazom bio značajno veći ($P<0,05$) nego u ostalim jatima.

Ključne riječi: influenca ptica, zarazni bronitis, mješovita zaraza