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Original Article

Molecular Detection of Avian Infectious Bronchitis Viruses in Live Bird Markets, Gilan Province

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ABSTRACT

Coronaviruses (AvCoV) which include infectious bronchitis virus (IBV) and other bird coronaviruses belong to the genus *gammacoronavirus*, subfamily *Coronavirinae*. One of the most prominent representatives of *gammacoronavirus* genus is infectious bronchitis virus (IBV) which is a highly contagious viral pathogen of chickens causing considerable economic losses to the poultry industry .IBVs mostly affect the respiratory, urinary, and reproductive tracts leading to a substantial drop in production. Backyard poultry in the villages usually share their food and water with free flight birds which puts them at serious risk of disease transmission. Furthermore, the poor hygienic measurements which are often used in backyard flocks make them a potential reservoir for diseases that can be transferred to commercial poultry flocks. Live bird markets (LBMs) which receive live poultry to be resold or slaughtered and sold onsite play a significant role in spreading infectious diseases among the different bird species. In the present study, a number of 354 cloacal swab samples were collected from different bird species from LBMs of Gilan province. Subsequently, after RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR) technique was carried out using specific primers of S1 gene to detect coronavirus infectious bronchitis virus. Two samples from backyard chickens were reported to be positive to coronavirus which were named Iran/Backyardchicken96/2017 and Iran/Backyardchicken94/2017. The results of the phylogenetic analysis demonstrated that these two isolates are placed in QX and IS-1494 strains, respectively. On a final note, the obtained results highlighted the role of live birds offered in LBMs in the epidemiology of IBV and the transmission of the virus to the industrial flock.

Keywords: Avian Infectious bronchitis, Phylogenetic analysis, Backyard poultry, Live bird market, Gilan Province

Détection Moléculaire des Virus de la Bronchite Infectieuse Aviaire dans les Marchés d'Oiseaux Vivants de la Province de Gilan

Résumé: Les coronavirus (AvCoV), qui incluent le virus de la bronchite infectieuse (VBI) et d'autres coronavirus d'oiseaux, appartiennent au genre *gammacoronavirus* et à la sous-famille des *Coronavirinae*. L'un des représentants les plus éminents du genre des *gammacoronavirus* est le virus de la bronchite infectieuse (VBI),un pathogène viral hautement contagieux touchant les poulets, causant des pertes économiques considérables à l'industrie aviaire . Les VBIs affectent principalement les voies respiratoires, urinaires et reproductives, entraînant une baisse substantielle de la production. Dans les villages, les volailles de basse-cour partagent généralement leur nourriture et leur eau avec des oiseaux en liberté, ce qui les expose à un risque sérieux de transmission de maladies. En outre, les mauvaises mesures sanitaires, courantes dans les troupeaux de basse-cour, en font des réservoirs potentiels pour des maladies transmissibles aux volailles commerciales.

Les marchés d'oiseaux vivants (MOVs) qui reçoivent des volailles vivantes à revendre ou à abattre jouent un rôle important dans la propagation des maladies infectieuses parmi les différentes espèces d'oiseaux. Dans cette étude, un total de 354 échantillons d'écouvillons cloacaux a été prélevé sur différentes espèces d'oiseaux des MOVs de la province de Gilan. Après l'extraction de l'ARN, la technique d'amplification en chaîne par polymérase, couplée à une transcription inverse (RT-PCR) a été réalisée en utilisant des amorces spécifiques du gène S1 pour détecter le VBI . Deux échantillons de poulets de basse-cour se sont avérés positifs pour le coronavirus, nommés Iran/Poulets de Basse-Cour96/2017 et Iran/Poulets de Basse-Cour96/2014. Les résultats de l'analyse phylogénétique ont démontré que ces deux isolats sont placés dans des souches QX et IS-1494, respectivement. Sur une note finale, les résultats obtenus ont mis en évidence le rôle des oiseaux vivants à la vente dans les MOVs dans l'épidémiologie du VBI et la transmission du virus aux troupeaux industriels.
Mots-clés: Bronchite infectieuse aviaire, analyse phylogénétique, volailles de basse-cour, marché aux oiseaux vivants, province de Gilan

INTRODUCTION

Coronaviruses (CoVs) are enveloped, positive-sense, single-stranded RNA viruses belonging to family *Coronaviridae*. There are four genera of coronaviruses, including *Alpha-, Beta-, Gamma-,* and *Deltacoronavirus* (Gonzalez et al., 2003). Avian coronaviruses (AvCoV) belong to *gammacoronavirus* genus, subfamily *Coronavirinae*, and contain infectious bronchitis virus (IBV) and other bird coronaviruses. AvCoV can cause diseases in Galliformes birds, such as chickens, turkeys, pheasants, peafowl, partridge, guinea fowl, quails, and wild birds with respiratory sings, as well as kidney diseases, undesirable egg quality, and mortality in some cases (Jackwood and de Wit, 2013). The most prominent representative of *gammacoronavirus* genus is infectious bronchitis virus (IBV) which is a highly contagious viral pathogen of chickens and incurs dramatic economic losses to the poultry industry (Cavanagh et al., 2002). The avian infectious bronchitis virus (IBV) which was first isolated in 1937 can be destructive and causes devastating economic losses in poultry industry worldwide and induces an acute, highly contagious upper respiratory tract disease in chickens and other fowls (Sjaak de Wit et al., 2011). IBVs mostly affect the respiratory, urinary, and reproductive tracts leading to a substantial drop in production (Worthington and Jones, 2006). IBV infections can also be concurrent with the secondary bacterial infections caused by *Escherichia coli*, *Mycoplasma gallisepticum, Mycoplasma synoviae,* and *Ornithobacterium rhinotracheale* (Van Empel et al., 1996; Naqi et al., 2001; Matthijs et al., 2003; Landman and Feberwee, 2004). Backyard poultry which are reared in the villages often stray into forest patches and share food and water sources with free flight birds; therefore, they will be at serious risk of disease transmission (Panshin et al., 2002; Peterson et al., 2002). Furthermore, the poor biosecurity measurements which are often used in backyard flocks make these flocks a potential reservoir for diseases that can transfer to commercial poultry flocks, especially the diseases which have become rare in these operations (Kelly et al., 1994; Willoughby et al., 1995) .Live poultry markets (LPMs) or live bird markets (LBMs) are the popular places all over the world that receive live poultry to be resold or slaughtered and sold on-site. These markets are usually located near residential areas in the cities (Senne et al., 2003). As illustrated by the results of some epidemiological studies, LPMs can play a key role in spreading the infectious diseases among the bird species; moreover, they can transfer some diseases, such as Avian Influenza, to humans (Nishiura et al., 2013; Zhou et al., 2015). The present study aimed to detect avian infectious bronchitis viruses in different

species of birds, such as backyard chicken, turkey, woodcock, and teal in the LBMs of Gilan province, north of Iran.

MATERIAL AND METHODS

Study population and Sampling. This cross-sectional survey was conducted during winter and fall of 2017 in a LBM of Gilan province, north of Iran. The LBM is located in an area with a high density of commercial poultry farms, including broiler and broiler breeders. A total number of 345 Cloacal swabs samples were collected from different bird species, including backyard poultry and migratory waterfowl, which were sold in this market. The sampled species included chickens (Gallus gallus), turkey (Meleagris gallopavo), Eurasian teal (Anas Crecca), common blackbird (Turdus merula), and Eurasian woodcock (Scolopax rusticola). Swab sampled were transferred to the Central Laboratory of Veterinary Medicine Faculty of Tehran University using cold chain.

RNA extraction. For RNA extraction, every five swab samples were pooled and RNA was extracted from swab samples using a commercial extraction kit (High Pure RNA Isolation kit, Roche). The extraction was performed based on the manufacturer's instructions. RNA extraction was carried out to all samples and the extracted RNA was stored at -70 °C until further usage.

cDNA synthesis. The reverse transcription was performed on the viral RNA using RevertAid first-strand cDNA synthesis (Thermo Scientific). The cDNAs were stored at -20 °C until subsequent use.

Quantitative real-time reverse transcription polymerase chain reaction. Real-time reverse transcription polymerase chain reaction (RT- PCR) was conducted on extracted RNAs in a QIAGEN Rotor-Gene Q (Corbett Rotor-Gene 6000) to detect coronavirus on these products. The primers which were designed based on the previous study to target highly conserved region of the 5-UTR (Callison et al., 2006) and Taqman® dual-labeled probe were used to amplify and detect a 143 bp fragment at the 5′UTR of the coronavirus genome (Table 2).

Partial S1 amplification for genotyping

A pair of degenerated primers (SX1, SX2, SX3, and SX4) (Table 2) were selected to be used in the initial PCR and the subsequent nested PCR (Najafi et al., 2016). First-round amplification was performed on a final volume of 20 µL (2 µL D.W, 13 µL Sinaclon 2X PCR master mix (Sinaclon, Iran), 2 µL of SX1 and SX2 primers, as well as 3 µL of cDNA. The amplification was performed with a thermal profile (94 °C for 2 min, 94 °C for 15 sec, 58 °C for 30 sec, 72 °C for 30 sec, and 72 °C for 10 min) for 35 cycles. Amplifications were carried out in an Eppendorf master cycler gradient thermocycler (Eppendorf, Hamburg, Germany). Nested-PCR reactions (20 uL) were performed using 1 uL of the first PCR product. The reaction mixture was the same as the previous PCR using nested primers (SX3 & SX4). The reaction products were analyzed by electrophoresis in 1.5% agarose gels in TAE buffer, stained with GelRed™ (Biotium, USA), and visualized under UV light.

Sequencing and bioinformatics analysis. The PCR products of all positive samples were purified using a commercial PCR Purification Kit (Bioneer, South Korea) and were sent for sequencing (Bioneer, South Korea). All sequences from a given sample were combined and used to construct alignments. ClustalX (Version 1.83) multiple sequence alignment analysis was performed to calculate the percentage of sequence similarity between our positive samples and the sequences of referral strains and other coronavirus strains.The phylogenetic trees of sequences were constructed using the neighbor-joining method and the Kimura 2-parameter model by MEGA package(version 5.1) (Tamura et al., 2011). A bootstrap resampling analysis was performed (1000 replicates) to test the robustness of the major phylogenetic groups. The positive samples are available in a genebank: MH194249-MH194250.

RESULTS

RNA extraction and molecular detection. From 345 Cloacal swabs samples, only two samples from backyard chickens were positive for avian coronavirus-specific gene elements (115 bp) and were named Iran/ Backyardchicken96/2017 and Iran/Backyardchicken94/ 2017.

Sequence analysis. The sequencing of the amplified region of the S1 gene of these two isolates was accomplished. Nucleotide sequence demonstrated 71.27-100% identity with 15 selected sequences of ACVs strains in the GenBank DNA (Table 1). Based on the results of phylogenetic analysis, the Iran/Backyardchicken96/2017 isolate was placed in QX strains. Moreover, there was 90.30% and 88.43% similarity between this isolate and Iran/QX/PCRLab and QX (AF193423), respectively (Figure 1). On the other hand, Iran/Backyardchicken94/2017 isolate was classified in IS-1494 strain (Variant2) and had 99.63% and 98.88% identity with Iran/IS-1494/UT-MG197/2017, Iran/IS-1494/UT-MG161/2017, and IS/1494/06 (HM131453) respectively (Figure 1). Furthermore, these two isolates showed 76.49% similarity in the nucleotide sequence of S1 gene (Table 1).

DISCUSSION

Gilan province enjoys a high status among the provinces of Iran and has one of the largest poultry population in the country accounting for about 10% of the total broilers meat production. Numerous villagers raise birds in their backyard, along with the industrial rearing of poultries. In addition, the temperate climate and the presence of numerous ponds have made Gilan the destination of many migratory birds from Central Asia and Russia. The high density of commercial, backyard, and migratory birds can lead to a transition of various diseases among them. The economy of rural families depends on keeping backyard poultries and hunting migratory waterfowl. Based on Iranian Ministry of Agriculture, 50 million broiler chickens are reared every year in Gilan, and 4.51 million backyard poultry are kept in northern provinces. In recent years, there have been numerous reports of highly pathogenic avian influenza (HPAI) outbreaks from the backyard and migratory birds in northern provinces, including Gilan. Therefore, Iranian Veterinary Organization (IVO) routinely inspects some of the viral diseases with high mortality among backyard and migratory birds.

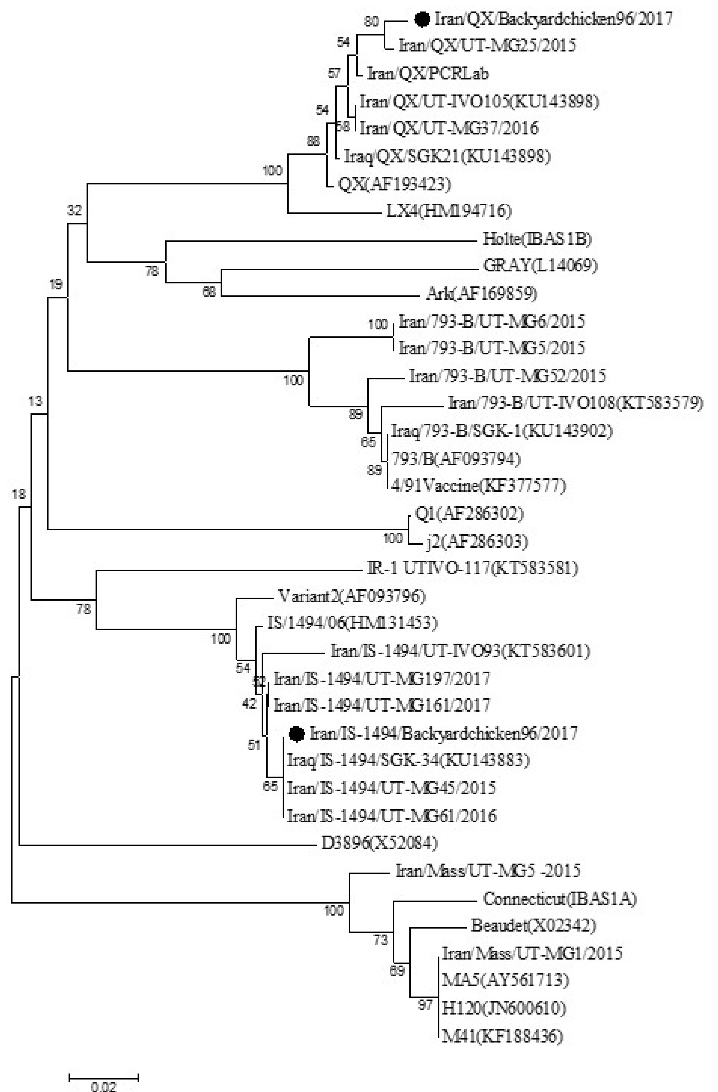

Figure 1. Phylogenetic tree of the nucleotide sequence of S1 gene reverse transcription polymerase chain reaction fragment of some Avian Coronavirus Viruses (ACVs) tested and of the published sequence in GenBank created by the neighbor-joining method with MEGA5 program. Values at the branches and clusters are bootstrap value and bar indicates distance scale from the roots.

Consequently, numerous studies were performed on phylogenetic or serological features of highly

pathogenic avian influenza (HPAI) and Velogenic Newcastle Disease (VND) in backyard and commercial poultry; nonetheless, the importance of IB is underestimated. The first isolation of IBV from chicken farms in Iran was reported in 1994 by Aghakhan et al. (Aghakhan et al., 1994). Numerous studies have been carried out on genotyping of IBV in commercial farms of Iran. As evidenced by the results of these studies, IBV strains isolated in Iran can be classified into seven distinct phylogenetic groups (Massachusetts, 793/B, IS-1494, IS-720, QX, IR-1, and IR-2) (Najafi et al., 2016). The most prevalent type is IS 1494-like, followed by 793/B (Modiri Hamadan et al., 2017). To the best of our knowledge, the present research is the first study on genotyping of IBV in the LBMs of Gilan. We found two molecular positive samples both of which were from backyard chickens and all samples collected from migratory birds were negative. Based on S gene sequencing, the isolates belonged to QX and IS 1494-like genotypes. Their sequences submitted to the GenBank with the accession number of MH194249 and MH194250. In a study that was conducted on backyard poultries in Mazandaran, another northern province of Iran, the infection rate was reported as 41.3% based on molecular surveillance and the dominant genotype was 793/B (Shokri et al., 2018). Unlike the results obtained in Mazandaran, as a neighboring province of Gilan, 793/B was not detected in our samples. Along the same lines, in a study on the serological presence of IB antibodies in backyard poultries of Isfahan province, central of Iran, 85.3% of samples were positive (Mahzounieh et al., 2006). The IBV seroprevalence in backyard poultries of different cities of Iran is highly variable (14-100%) with a higher prevalence in cities with more commercial poultry farms (Shokri et al., 2018). There is no commercial vaccine of these

Table 1. Percent identity of nucleotide sequences of the S1 gene of present isolates, as compared to the sequences from GenBank. Iran/Backyardchicken96/2017 and Iran/Backyardchicken94/2017 were the isolates of the current study.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	Iran/Backyardchicken96/2017																		
2	Iran/Backyardchicken94/2017	76.49																	
3	Iran/IS-1494/UT-MG197/2017	76.87	99.63	82.09															
4	Iran/IS-1494/UT-MG161/2017	76.87	99.63	82.09	100.00														
5	Iran/QX/UT-IVO105(KU143898)	89.93	83.21	98.51	83.58	83.58													
6	Iran/QX/PCRLab	90.30	82.84	98.88	83.21	83.21	99.63												
7	Iran/793-B/UT-MG6/2015	76.12	83.58	82.09	83.96	83.96	82.84	83.21											
8	793/B(AF093794)	76.12	83.21	82.46	83.58	83.58	83.21	83.58	95.90										
9	4/91Vaccine(KF377577)	76.12	83.21	82.46	83.58	83.58	83.21	83.58	95.90	100.00									
10	IS/1494/06(HM131453)	76.49	98.88	81.72	99.25	99.25	83.21	82.84	83.96	83.58	83.58								
11	QX(AF193423)	88.43	83.96	97.01	84.33	84.33	98.51	98.13	83.58	83.58	83.58	83.96							
12	LX4(HM194716)	86.19	82.46	93.28	82.84	82.84	94.03	93.66	82.46	81.72	81.72	82.46	95.52						
13	Q1(AF286302)	73.88	83.21	79.48	83.58	83.58	80.60	80.60	81.34	81.72	81.72	83.58	81.34	79.10					
14	H120(JN600610)	71.27	82.46	77.99	82.84	82.84	79.10	79.10	77.61	77.24	77.24	82.46	80.22	80.22	78.36				
15	M41(KF188436)	71.27	82.46	77.99	82.84	82.84	79.10	79.10	77.61	77.24	77.24	82.46	80.22	80.22	78.36	100.00			
16	Ark(AF169859)	75.00	81.72	82.09	82.09	82.09	82.84	83.21	81.72	80.60	80.60	82.09	83.96	83.96	81.34	81.72	81.72		
17	Connecticut(IBAS1A)	71.27	80.60	77.99	80.97	80.97	79.10	79.10	77.61	77.99	77.99	80.60	80.22	79.48	76.87	96.27	96.27	80.22	

Table 2. Primers and probe used to amplify and detect the coronavirus genome

Forward primer	IBV5′GU391 5′-GCTTTTGAGCCTAGCGTT-3′
reverse primer	IBV5′GL533 5′GCCATGTTGTCACTGTCTATTG-3′
probe	IBV5′G probe 5′-FAMCACCACCAGAACCTGTCACCTC-BHQ1-3′
SX1	CACCTAGAGGTTTGYTWGCATG
SX2	TCCACCTCTATAAACACCYTTAC
SX3	TAATACTGGYAATTTTTCAGATGG
SX4	AATACAGATTGCTTACAACCACC

genotypes available in Iran; therefore, these isolates can not be due to vaccination. With about 70% prevalence, IS-1494 like genotype is the most prevalent genotype of IBV in Iran poultry farms (Modiri Hamadan et al., 2017). IS 1494-like IBV was first reported in Middle East (Israel) in 2006 (Gelb et al.,
2005); thereafter, IS 1494-like IBV spread rapidly in the region and was detected in Jordan, Turkey, and many other Middle Eastern countries (Ganapathy et al., 2015). This genotype was detected in Iran for the first time in 2010 (Hosseini et al., 2015); subsequently, IS 1494-like became the most dominant genotypes in this country (Najafi et al., 2016). It is the first time that IS 1494- like genotype has been isolated from backyard poultry in Iran which highlights the importance of controlling the IB infection in backyard poultries in infectious disease prevention and control in commercial farms. IS 1494-like isolate (Iran/IS-1494/ Backyard chicken94/2017) showed marked similarities (98.88-99.63%) with previous Iranian IS-1494 isolates. Another isolate belonged to QX genotype. QX type IBV was first isolated by Bozorgmehri Fard in Iran (Najafi et al., 2016). Its prevalence decreased from 10% of all molecular detected isolates within 2014-2015 to 2.75 within 2015-2017 (Modiri Hamadan et al., 2017). This may be due to changing vaccination program to combine Massachusetts and 793/B type IB vaccines. The QX isolate of the present study demonstrated 90.30% and 88.43% similarity with Iran/QX/PCRLab and QX (AF193423) isolates, respectively.

The presence of IBV in backyard chickens of Gilan underlines the importance of controlling IB in backyard poultries. Commercial poultries have always received special attention, while backyard poultries have suffered serious neglect. It is necessary for farms near rural areas to improve their biosecurity. In regions, such as Gilan, where poultry rearing is a major source of income for villagers, commercials farms located in the vicinity of villages lead to IBV circulation in backyard poultries which are easily transmitted to neighboring farms despite vaccination. It is necessary to monitor IB, HPAI, and VND in backyard poultries and migratory birds to predict future outbreaks in commercial farms and design IB vaccination program not only based on genotypes circulated in commercial farms but also prevalent genotypes in backyard poultries.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Authors' Contribution

Study concept and design: Rezaee, H.
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Administrative, technical, and material support: Esmaeelzadeh dizaji, R.; Motamed Chaboki, P.

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