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Detection and Molecular Characterization of Gammacoronavirus in Quail Population in Iran

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Abstract

BACKGROUND: Gammacoronaviruses, which are single-stranded, positive-sense RNA viruses, are responsible for a wide variety of existing and emerging diseases in birds. The Gammacoronaviruses primarily infect avian hosts.

OBJECTIVES: This study aimed to investigate the genetic diversity of Gammacoronaviruses in quail population in Iran.

METHODS: In the period from 2016 to 2018, samples from 47 quail flocks with or without enteric signs, were collected from four provinces in Iran.

RESULTS: Gammacoronavirus was detected in samples of 4 flocks by using RT-PCR and characterized by N gene sequencing. The isolates formed a distinct group from other Gamma- coronaviruses groups

CONCLUSIONS: The finding suggests the existence of a novel Gammacoronavirus circulating in quail farms. The phylogenetic relationship of the isolates concerning different sequences and geographical regions displayed complexity and diversity. The present study is the first detection of Gammacoronavirus in quail farms in Iran. Further studies are required and should include the isolation and experimental studies of Gammacoronaviruses in Iran.

KEYWORDS: Bird, gammacoronavirus, N gene, phylogenetic relationship, quail

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Introduction

The majority of emerging infectious diseases of poultry are caused by RNA viruses. Their specific characteristics like high rates of mutation, short generation time and large population sizes help these viruses very much in rapid evolution (Jackwood et al., 2012).

The Coronaviridae is now divided into two subfamilies as Coronavirinae and Torovirinae. Coronaviruses (CoVs) are enveloped viruses within the Coronaviridae family (Jackwood and de Wit, 2013) and based on the genome size and genetic complexity are the largest RNA viruses identified so far. According to the latest update of the International Committee for the Taxonomy of Viruses (ICTV), Coronaviruses (CoVs) are classified into four genera as Alphacoronavirus (αCoV), Betacoronavirus (βCoV), Gammacoronavirus, and Deltacoronavirus (Adams & Carstens, 2012). Two-thirds of the genome in the 5′ end is occupied by two overlapping open reading frames encoding viral RNA-dependent RNA polymerase (RdRp). At the 3′ end, genes encoding the four major structural proteins including spike (S), envelope (E), matrix (M), and nucleocapsid (N) along with genes 3 and 5 which encode nonstructural accessory proteins are located (Britton et al., 2006; Liu et al., 2009).

Alphacoronaviruses and Betacoronaviruses have been isolated from several mammal species, including humans, dogs, cats, and cows. However, all known Gammacoronaviruses only infect avian species with some exceptions. Examples are chicken infectious bronchitis virus (IBV), turkey coronavirus (TCoV) and pheasant coronavirus (Jonassen et al., 2005). Meanwhile, infections in several other species including greylag geese, mallard ducks, pigeons (Cavanagh et al., 2002) and quail (Coturnix japonica)) have been reported, often with different tissue tropism (Torres et al., 2013; Torres et al., 2016). These findings together with the isolation of Gammacoronaviruses from several other avian species caused the experts to suspect a role for some species like quail as CoV reservoirs and CoV carriers influencing IBV epidemiology.

Mutations and recombination in the CoVs genome have resulted in viruses with different tissue tropism, increased virulence, and increased ability to persist in the chickens. (Jackwood et al., 2012). Gammacoronaviruses, genetically related to IBV, were detected in healthy galliform and non-galliform avian species. This finding may suggest that wild birds might carry IBV-like viruses asymptomatically (Hughes et al., 2009) and prompted us to study the surveillance of Gammacoronavirus in quail farms.

The aims of this study were to detect the presence of Gammacoronavirus in quail population in different areas of Iran and preliminary molecular characterization of obtained avian Gammacoronaviruses.

Materials and Methods

Sampling: A total of 884 cloacal/fecal swabs together with kidney, oviduct, respiratory and intestinal tract samples from 47 quail flocks were collected from November 2016 to June 2018. The sample size of each flock was determined according to the size of that flock. Samples were provided from Yazd, Alborz, Ghazvin, and Tehran provinces.

Virus isolation and Propagation: All samples were homogenized, and a 10% (w/v) suspensions were made in PBS. Subsequently, samples were centrifuged at 1500× g for 20 min at 4 ºC. The supernatant was used to inoculate fertile specific-pathogen-free (SPF) embryonated chicken eggs. Homogenized tis-

sue samples supplemented with 10,000 IU of penicillin, 10,000 IU of streptomycin and 250 IU of amphotericin B were used for this isolation. After one hour at room temperature, 200 µl aliquots of the homogenate of each sample was inoculated into the allantoic cavity of five 9 to 11-day-old SPF embryonated chicken eggs. The inoculated eggs were incubated at 37 ºC and candled daily to check embryonic viability. Two to 3 days post ncubation, the allantoic fluid was harvested and used for subsequent passages. Six serial passages were performed and the allantoic fluid was collected 48–72 h post-inoculation. Three uninoculated SPF eggs were also used as controls in each isolation process (Ghalyanchi Langeroudi et al., 2017).

RT-PCR: Total RNA was extracted from 250 µl individual or pooled supernatants using the High pure RNA extraction kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. The purity of the extracted RNA was determined by considering the ratio of the readings at 260 and 280 nm. The extracts were stored at -20 ºC until future use. The cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) as recommended by the manufacturer. The assay was standardized using Qiagen RT-PCR kit and master mix recipes were prepared according to the manufacturer's instructions. The assay was performed in a final volume of 25 µl containing 2 µl RT-PCR master mix (HotStarTaq DNA polymerase and 4 mM MgCl2), 0.5 µl of RT mix and 400 nM of PCR primer. The target gene for PCR amplification was nucleocapsid (N) segment of Gammacoronaviruses genome. For N gene, forward (N103F): cctgatggtaatttccgttggg and reverse (N102R): acgcccatccttaataccttcctc primers were used (Loa et al., 2006). Prior to amplification, the RNA was transcribed at 50 ºC for 30 min. Then, the process was followed by one cycle of 94 ºC for 15 min to activate and inactivate HotStarTaq DNA polymerase and reverse transcriptase, respectively. The thermocycler program included 40 cycles of denaturation at 94 ºC for 15 s and annealing at 58 ºC for 30 s; and a final extension at 72 ºC for 45 s. The PCR products were electrophoresed on 1.5% agarose gels in Tris/Borate/EDTA (TBE) buffer, stained with GelRedTM (Biotium, USA), visualized under UV light and photographed. Furthermore, all positive samples were tested by using RT-PCR to detect the UTR gene as previously described (Ghalyanchi Langeroudi et al., 2017). Attempts were also made to amplify the S gene of viruses detected in this study by trying several primers as cited in previous literatures (Seger et al., 2016; Hamadan et al., 2017).

Sequence and phylogenetic analysis: The RT-PCR products were purified using the PCR product purification kit (Roche Diagnostics, Germany) according to the manufacturer's instruction and submitted for automated sequencing at the Bioneer Company using PCR primers as sequencing primers. The sequences were aligned and analyzed by BioEdit software ver. 7.0.9.0 (Hall, 1999) and DNASIS MAX 3.0 (Hitachi Solutions America), and compared with selected sequences available in GenBank database. The genetic distances of the aligned sequences were analyzed with MEGA 7.0 using the neighbor-joining method with the maximum-likelihood model (Kumar et al., 2016). The sequence data from four virus isolates of the present study were submitted to the GenBank and the following accession numbers were assigned: MK183095, MK183096, MK183097 and MK183098 for UT-BPG1, UT-BPG2, UT-BPG3 and UT-BPG4 virus isolates, respectively.

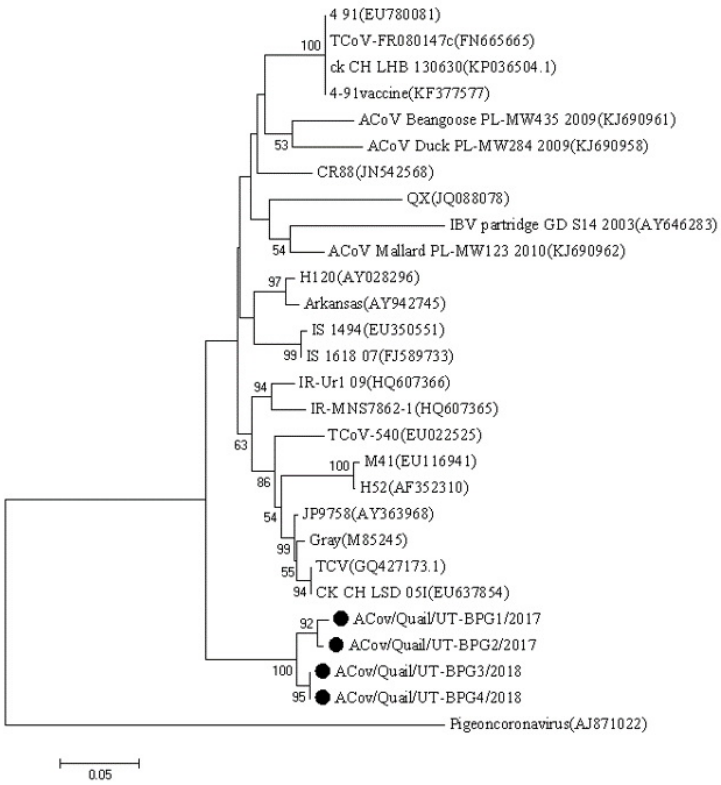

Figure 1. Phylogenetic tree based on sequences of N gene. The neighbor-joining method was used. Black circles indicate viruses detected in the present study.

Results

From 2016 to 2018, 47 flocks were examined in which 4 (8.5%) positive flocks were found, all located in Tehran province. According to the phylogenetic analysis of N gene, the viruses were clustered in a distinct group other than the known Gammacoronaviruses groups (Fig. 1). The similarity of the sequences observed in the current study and those of the other Gammacoronaviruses are presented in Table 1. The findings of this study showed the lowest similarity (97.24%) between ACov/Quail/UT-BPG2/2017 and ACov/Quail/UT-BPG3/2017; and the highest similarity (100%) between ACov/Quail/UT-BPG3/2018 and ACov/Quail/UT-BPG4/2018. The isolates were most similar to IR-Ur1_09 (87.34%) and IS_1618_07 (88.59%). Besides, the similarity to 4/91 vaccine virus varies from 85.74 to 86.64 while it was from 85.16 to 86.47 for H120 vaccine virus. All positive samples were also positive for UTR gene. However, our attempts to amplify the S gene of the four virus isolates of this study failed.

Discussion

In this study, Gammacoronavirus was detected in 4 flocks of quail, two of which were presented with mild enteritis and depression. The virus was detected in kidney, reproductive tract, and content of intestine. This suggests the fecal-oral route as the possible route of transmission similar to chickens (Torres et al., 2013).

To our knowledge, there has been no record of vaccination against IBV in any flocks

Table 1. Identity percentage of the N gene nucleotide sequences of Gammacoronaviruses detected from Iran and other Gammacoronaviruses.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	(TCV (GQ427173.1																						
2	(Gray (M85245	98.64																					
3	(H120 (AY028296	93.27	93.28																				
4	(M41 (EU116941	93.21	93.59	88.18																			
5	(IR-Ur1_09 (HQ607366	94.38	95.11	93.61	90.58																		
6	IR-MNS7862-1 ((HQ607365	92.89	93.64	92.86	88.59	96.51																	
7	(H52 (AF352310	93.59	93.97	88.59	99.66	90.98	89.00																
8	(IS_1494 (EU350551	91.74	91.77	94.72	87.35	91.29	90.52	87.77															
9	(IS_1618_07 (FJ589733	92.13	92.15	95.09	87.77	91.69	90.92	88.18	99.66														
10	(4-91vaccine (KF377577	90.26	90.65	90.56	88.67	91.37	90.99	88.67	89.40	89.81													
11	(QX (JQ088078	84.58	85.43	88.18	81.39	85.67	85.70	81.85	87.03	87.44	85.33												
12	(EU780081) 91_4	90.26	90.65	90.56	88.67	91.37	90.99	88.67	89.40	89.81	100.00	85.33											
13	(TCoV-540 (EU022525	93.97	94.72	90.99	92.10	92.50	92.48	92.48	89.81	90.20	90.65	83.71	90.65										
14	IBV-partridge-GD-S14-2003 (AY646283)	81.59	82.04	81.14	81.53	82.85	81.99	81.99	84.62	85.04	82.93	80.22	82.93	82.04									
15	TCoV-FR080147c (FN665665)	90.26	90.65	90.56	88.67	91.37	90.99	88.67	89.40	89.81	100.00	85.33	100.00	90.65	82.93								
16	ACoV-Beangoose-PL-MW435_2009 (KJ690961)	89.07	89.07	90.17	84.39	89.38	89.00	84.84	88.18	88.59	87.40	81.76	87.40	86.61	81.46	87.40							
17	ACoV_Mallard_PL-MW123_2010 (KJ690962)	90.26	90.65	89.46	87.84	90.60	91.39	88.25	90.25	90.64	89.87	88.61	89.87	89.08	88.20	89.87	89.02						
18	ACoV_Duck_PL-MW284_2009 (KJ690958)	86.29	86.70	87.37	84.60	89.47	88.68	84.60	86.16	86.59	93.23	82.34	93.23	86.29	81.62	93.23	91.78	87.89					
19	Pigeoncoronavirus ((AJ871022	52.52	53.17	53.17	51.25	53.72	51.77	51.25	54.45	53.81	51.18	45.10	51.18	53.81	49.81	51.18	50.50	53.84	49.16				
20	ACov/Quail/UT-BPG1/2017	84.87	85.36	85.16	82.34	87.35	84.80	81.89	87.32	87.74	85.79	77.21	85.79	85.79	78.45	85.79	85.74	85.01	87.03	50.54			
21	ACov/Quail/UT-BPG2/2017	85.24	85.74	85.52	82.72	87.71	85.16	82.26	87.67	88.09	85.74	77.60	85.74	86.16	78.85	85.74	86.10	85.39	87.40	52.48	98.98		
22	ACov/Quail/UT-BPG3/2018	86.16	86.63	86.47	83.68	88.59	86.10	83.24	87.74	88.15	86.63	78.68	86.63	87.05	79.85	86.63	86.16	86.27	87.86	53.79	97.59	97.24	
23	ACov/Quail/UT-BPG4/2018	86.16	86.63	86.47	83.68	88.59	86.10	83.24	87.74	88.15	86.63	78.68	86.63	87.05	79.85	86.63	86.16	86.27	87.86	53.79	97.59	97.24	100.00

of our investigation. The flocks were only vaccinated against Newcastle disease. The current study viruses were phylogenetically distinct from infectious bronchitis virus, and as the phylogenetic analysis results showed, all Gammacoronavirus isolates detected in quails were different from IBV vaccines. In case of the similarity to vaccine strains, our viruses were 85.74-86.64 and 85.16-86.47 percent similar to 4/91 and H120 vaccine strains, respectively.

Currently, RT-PCR amplification of the S1 gene followed by nucleotide sequence analysis is being used as the molecular typing test to inexpensively detect the genetic type of some viruses in a short period of time. Unfortunately, all of our attempts to amplify the S gene of viruses detected in this study were unsuccessful despite trying several primers (Seger et al., 2016; Hamadan et al., 2017). Some coronaviruses like infectious bronchitis virus grow in allantoic sac of chicken embryonated egg, but all of our attempts to isolate Gammacoronaviruses failed (Jackwood and de Wit, 2013). Therefore, the discovery of new viruses using Next Generation Sequencing (NGS) technology including DNA and RNA sequencing in recent years might help us to improve our knowledge about Gammacoronaviruses.

To our knowledge, the present study is the first detection of Gammacoronavirus in commercial quails in Iran and because of the potential to act as a host reservoir for Gammacoronavirus, with possible transmission to chicken, studying infectious bronchitis virus in quails seems necessary. These findings not only reveal a prevalence of Gammacoronaviruses circulating in birds other than chickens but also suggest a potential role for spreading viruses.

Based on N gene nucleotide sequences, no sample was placed in the same group with previously published sequences of Gammacoronavirus isolates. While the most similar isolates with 100% similarity were isolates ACov/Quail/UT-BPG3/2018 and ACov/Quail/UT-BPG4/2018, only 97.24% similarity were found between isolates ACov/Quail/UT-BPG2/2017 and ACov/Quail/UT-BPG3/2017. This study may provide preliminary information on the molecular epidemiology of Gammacoronavirus in quail population of Iran. Additional Gammacoronavirus surveillance and full genome sequences using next-generation sequencing would better clarify the characteristics and the origin of Gammacoronavirus isolates obtained in this study.

These avian Gammacoronaviruses might not cause severe illness in their hosts, hence; it may easily become endemic in avian population. However, Circella et al. (2007) reported the coronavirus associated with an enteric syndrome in a quail farm. It was detected by electron microscopy and RT-PCR in the feces and intestinal content of the dead quails. Their investigation showed that S1 portion of those isolates displayed 16% to 18% amino acid identity with IBV, and 79% to 81% identity with turkey coronavirus. In this study, the quails of two flocks showed severe diarrhea, reduced growth and depression. Isolation and inoculation of quail Gammacoronavirus in an experimental condition will help us to confirm the pathogenicity of these novel viruses in quails.

In another study, Torres et al. (2017) detected Gammacoronavirus and Deltacoronavirus in quails and pheasants in Italy. Sequencing of S gene showed that quail's isolate was related to 793B and the isolate from pheasant was related to Mass type. In the present study, we did not try to detect Deltacorona-

virus, however: it seems necessary to investigate Deltacoronavirus in quail's population of Iran.

The findings of this study demonstrated that quails are major reservoirs for a wide range of Gammacoronaviruses. Due to intensive trading and uncontrolled movement of poultry and people between provinces of Iran, the distribution of Gammacoronavirus around poultry farms is highly possible. Constant updating of the data from continuing molecular surveillances of avian coronaviruses would complete this strange puzzle and also help us to be prepared for the possible emerging new variants.

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Conflicts of interest

The author declared no conflict of interest.

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مجله طب دامی ایران، ۱۳۹۷، دوره ۱۳، شماره ۳، ۲۵۹-۲۵۱

ردیابی و مطالعه ملکولی گاماکروناویروس ها در جمعیت بلدرچین های ایران

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چکیده

زمینهٔ مطالعه: گاماکروناویروس ها ، RNA ویروس های تک رشته ای و سنس مثبت هستند که سبب بیماری های متعددی در پرندگان می شوند.

هدف: هدف از این مطالعه بررسی تنوع ژنتیکی گاماکروناویروس ها در جمعیت بلدرچین های ایران بود

روش کار: هدف از این مطالعه بررسی تنوع ژنتیکی گاماکروناویروس ها در جمعیت بلدرچین های ایران بود.

نتایج: گاماکروناویروس در نمونه های جمع آوری شده از ۴ گله متفاوت بر اساس روش RT-PCR مثبت تشخیص داده شد و ژن N جدایه ها نیز تعیین توالی گردید. جدایه ها گروهی متمایز از سایر گاماکروناویروس ها تشکیل دادند.

نتیجه‌گیری نهایی: نتایج مطالعه حاضر وجود یک گاماکروناویروس جدید در چرخش در بین جمعیت بلدرچین های ایران را تایید می کند. ارتباط شجره شناسی جدایه ها با سایر جدایه ها از مناطق مختلف جغرافیایی، پیچیدگی و تنوع را نشان می دهد. مطالعه حاضر، اولین شناسایی گاماکروناویروس ها در جمعیت بلدرچین های تجاری در ایران می باشد. مطالعات تکمیلی از جمله جداسازی ویروس و مطالعات تجربی ضرروری بنظر می رسد.

واژه‌های کلیدی:

پرنده، گاماکروناویروس، ژنN، بلدرچین، ارتباط شجره شناسی

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