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Original Article

Molecular Detection of Gamma Coronaviruses in Bird Parks of Iran

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ABSTRACT

Gamma Coronaviruses (GCoVs) are distributed worldwide, affecting a wide range of bird species, the beluga whale, and bottlenose dolphins. Because of the limited proofreading capability in the viral encoded polymerase, they emerge genetically diverse. There has been no molecular surveillance data to describe the epidemiology of GCOVs in avian species. The present study was conducted to detect GCOVs in Tehran birds' parks, 2015. Cloacal swabs (267 samples) from eight different bird species ((Chickens (*Gallus gallus*), Pheasant (*Phasianus colchicus*), Turkey (*Meleagris gallopavo*), Partridge (*Perdix perdix*), Quail (*Coturnix coturnix*), Duck (Anas platyrhynchos), Goose (Anserini),and Guinea fowl (Numididae)) were collected, the viral RNA was extracted, the RT-PCR was performed using QIAGEN one step RT-PCR kit and the primers targeting "3'-UTR" and "Nucleocapsid" genes. The detection rate was approximately 8.99%. GCOVs were detected in the chicken, quail, pheasant, turkey, and the partridge with different prevalence rates. Phylogenetic tree based on partial nucleotide sequences of the N gene clustered the samples into two groups. It is the first report of GCOVs in non-commercial birds in Iran. According to our results, GCOVs are circulating in different avian species, and further studies are needed to isolate these viruses and evaluate their pathogenesis.

Keywords: Gamma coronavirus, Molecular detection, Bird Parks, Iran, Phylogenetic Analysis

Détection moléculaire du coronavirus gamma dans les parcs ornithologiques d'Iran

Résumé: Les coronavirus gamma (GCoV) sont répandus dans le monde entier et touchent un large éventail d'espèces d'oiseaux, ainsi que le béluga et le grand dauphin. Les polymérases codées par des virus sont génétiquement diverses en raison d'une capacité de relecture limitée. Il n'y a pas de données de surveillance moléculaire pour décrire l'épidémiologie des GCoV chez les espèces aviaires. La présente étude visait à détecter les GCoVs dans les parcs ornithologiques de Téhéran, en Iran, en 2015. Initialement, des écouvillons cloacaux (267 échantillons) ont été collectés chez huit espèces d'oiseaux, à savoir le poulet (*Gallus gallus*), le faisan (*Phasianus colchicus*), la dinde (*Meleagris gallopavo*), la perdrix (*Perdix perdix*), la caille (*Coturnix coturnix*), le canard (*Anas platyrhynchos*), d'oie (Anserini) et de pintade (Numididae). Ensuite, l'ARN viral a été extrait et la réaction en chaîne de la polymérase par transcription inverse (RT-PCR) a été réalisée à l'aide d'un kit QIAGEN de RT-PCR en une étape et des amorces ciblant les 3 gènes de la région non traduite (UTR)

et de la nucléocapside (N). Selon nos résultats, le taux de détection a été estimé à 8,99% et des GCoVs ont été trouvés chez les poulets, les cailles, les faisans, les dindes et les perdrix avec des taux de prévalence différents. L'arbre phylogénétique établi à partir des séquences de nucléotides partielles du gène N a divisé les échantillons en deux groupes. Aucune étude n'a été réalisée sur les GCoVs chez des oiseaux non commerciaux en Iran. Ces résultats montrent que les GCoVs sont prévalents parmi différentes espèces d'oiseaux. Cependant, des études supplémentaires sont nécessaires pour isoler ces virus et évaluer leur pathogenèse.
Mots-clés: Coronavirus gamma, Détection moléculaire, Parc ornithologique, Iran, Analyse phylogénétique

INTRODUCTION

Coronaviruses (CoVs) have positive-sense single-stranded RNA and belong to the *Coronaviridae* family in the *Nidovirales* order. The CoVs are the largest (27-32 kb long) non-segmented RNA viruses. The 5' two-thirds of the genome contains two large open reading frames (i.e., ORF1a and ORF1b), which encode the replicase complex. Once the virus enters the cells, a 3'-coterminal nested set of six mRNAs is generated (Muradrasoli et al., 2009). Subsequently, polyproteins 1a and 1a/b are translated from two overlapping open reading frames (mRNA1), encompassing approximately 74% of the genome at the 5' end. Subgenomic mRNAs (sgRNAs) 2, 3, 4, and 6 encode four structural proteins, namely the spike (S) glycoprotein, envelope (E) glycoprotein, membrane (M) glycoprotein, and nucleocapsid (N) protein, respectively (Liu et al., 2005). The CoVs are subdivided into four genera, namely alpha, beta, gamma, and delta coronaviruses (DCoVs). The CoVs found in avian species mainly belong to the genus of GCoVs; however, a debate has arisen around some isolates classified as DCoVs (Jordan et al., 2015). There is evidence regarding the identification of GCoVs, which are genetically related to avian infectious bronchitis virus (IBV), in healthy galliform and non-galliform birds. This finding suggests the possibility that wild birds can carry IBV-like viruses asymptomatically. Other studies have detected GCoVs that are genetically distinct from IBV in wild birds, including graylag goose (*Anser anser*), rock dove (*Columba livia*), mallard (Anas Platyrhynchos), Chinese bulbul (*Pycnonotus sinensis*), red-whiskered bulbul (*Pycnonotus jocosuss*), grey-backed thrush (*Turdus hortulorum*), blackbirds (*Turdus merula*), white-rumped munia (*Lonchura striata*), and scaly-breasted munia (*Lonchura punctulata*) (Hughes et al., 2009). The GCoVs have also been identified in mammals, such as beluga whale and Asian leopard cat (Dong et al., 2007; Mihindukulasuriya et al., 2008). Detection of RNA viruses is the first step toward treatment, control, and prevention of human and animal infectious diseases, which would be too difficult if not recognized early (Chen et al., 2013). There are scarce data regarding the genome sequence of IBV-like viruses in domestic fowls. For example, only one duck with IBV-like virus genome sequence has been submitted to the GenBank (Chen et al., 2013). Wild birds serve as reservoirs for several viruses, the most prevalent of which is the avian influenza A virus (Dong et al., 2007). Detection of CoV through electron microscopy and reverse transcription-polymerase chain reaction (RT-PCR) in the feces of the dead quails revealed an enteric syndrome associated with CoV. The *S1* gene sequence analysis of the CoV spike protein detected in quail species revealed 16-18% amino acid identity with IBV, and 79-81% identity with turkey coronavirus (TCoV) (Circella et al., 2007). Recently, Torres et al. (2016) have detected GCoV and DCoV in quails and pheasants (non-vaccinated) in Northern Italy. According to the *S* gene sequences, the

avian coronavirus (AvCoV) obtained from the quails and pheasants are correlated to 793B and Massachusetts (Mass) type IBV, respectively. On the other hand, based on the analysis of RNA-dependent RNA polymerase (RdRp), quail is susceptible to DCoVs (Torres et al., 2016). Moreover, the results of the study mentioned above demonstrated the presence of GCoVs and DCoVs in quails in vaccinated (Mass type) flocks in São Paulo state, southeastern Brazil, in 2013. Partial *S* gene analysis categorized the GCoVs in quail and layers with the Brazilian type. It was concluded that the Mass-type vaccination is not effective against IBV in quail species (Torres Alejo et al., 2016). According to the results of another study performed by the same researcher, based on the DNA sequences for the 3' untranslated region (UTR) and the gene encoding the RdRp, this AvCoV in quails with respiratory and reproductive signs is an IBV-like GCoV (Torres et al., 2013). Glycan array analyses revealed that *S1* proteins of enteric GCoVs, unlike the S1 proteins of IBV, recognize a unique set of non-sialylated type 2 poly-N-acetyl-lactosamines. Protein histochemistry results, along with those of pathogenicity analysis, demonstrate the limited tropism of S1 proteins to intestinal tissues of each particular host in turkeys, quails, and guinea fowls CoVs (Wickramasinghe et al., 2015). The CoVs harbored by pheasants are genetically close to IBV and TCoV, which share more than 95% sequence similarities in corresponding regions. As observed in different IBV serotypes, some viruses obtained from pheasants were 24% different from each other. The pheasant viruses have the *3* and *5* genes as well, which are also present in IBV, but not in the transmissible gastroenteritis virus and murine hepatitis virus groups of mammalian CoVs. Nucleotide sequences of genes *3* and *5* obtained from pheasants were 90% similar to those of CoVs from turkeys and chickens (Cavanagh et al., 2002). Based on *N* gene nucleotide sequences, no sample was placed in the same group with the previously published sequence of GCoV isolates. Regarding *N* gene sequences, virus isolates from turkeys in this study and also unpublished data from industrial poultry farms were close to IBV, which could be either due to infection with IBV-like viruses or the deficiency of previously submitted genes. Isolates from quails formed a separate group, associated with variant 2 (IS-1494) strain and 793/B. However, the final judgment should be based on *S* gene sequences. The present study provides basic information on molecular epidemiology and the detection of GCoVs in Iran. The results of this study could be used in more comprehensive research on different bird species in the country. Since DCoVs have been recently detected in different species of birds, DCoV detection is also important to make the study more comprehensive. Also, CoV isolation from birds' species using accurate methods helps understand their pathogenicity. Different prevalence rates of CoVs obtained from wild birds could be due to their various geographical regions and time of sample collection. About the ongoing emergence of new viral variants, continuous surveillance and molecular epidemiological studies are needed to provide data on viral evolutionary behavior. Possibly, IBV-like CoVs in wild birds is originated from IBVs in poultry. Wild birds may act as CoV gene carriers, even in a vast geographic territory. Also, new variants could arise from recombination occurring among different CoVs infecting the same wild bird host. Epidemiology, evolution, and genetics of some poultry CoVs are well characterized. However, there is a lack of information about the features of CoVs in wild birds. With this background in mind, the present study was conducted to detect GCoVs in the bird parks located in Tehran, Iran, in 2015-16.

MATERIAL AND METHODS

Sampling. For the purposes of the study, 267 cloacal swabs were collected from eight bird species, namely chicken (*Gallus gallus*), pheasant (*Phasianus colchicus*), turkey (*Meleagris gallopavo*), partridge (*Perdix perdix*), quail (*Coturnix coturnix*), duck (*Anas platyrhynchos*), goose (*Anserini*), and guinea fowl

(*Numididae*). These birds were taken from three bird parks located in the northeast, center, and west of Tehran Province, Iran. Scientific names of the birds are not used in the rest of the research.

RNA Extraction. The RNAs from the swab samples were extracted using a High-pure RNA extraction kit (Roche, Germany) according to the manufacture instructions. The purity of the extracted RNA was determined through the ratio of the readings at 260 and 280 nm. Negative control was used during the extraction procedure, and the RNA was stored at -70 °C until used.

One-step reverse transcription-polymerase chain reaction for detection of gamma coronavirus. The one-step RT-PCR kit (Qiagen) was used to amplify the viral genome. The total volume of the reaction was 25 µl, containing 2 µl RT-PCR master mix (HotStarTaq DNA polymerase and 4 mM $MgCl_2$), 0.5 µl of RT mix, and 400 nM of each PCR primers (Multiplex). The target genes of primers were *N* and 3'-UTR segments of GCoVs genome. The details of the primers are presented in Table 2. The RNA transcription was performed at 50 ℃ for 30 min, followed by incubation for 15 min in 94 ℃ to activate the HotStarTaq DNA polymerase and inactivate the reverse transcriptase. The thermal condition included 40 cycles of denaturation at 94 ℃ for 15 sec, annealing at 58 ℃ for 30 sec, and extension at 72 ℃ for 45 sec. Amplified PCR products were visualized on a 2% agarose gel stained with RedSafe™ stain to confirm their size. The NTC and at least one positive control RNA were used in each reaction.

Phylogenetic analysis. The PCR products were purified using the AccuPrep®PCR Purification Kit (Bioneer Co., Korea). The primers used in PCR were submitted for sequencing (Bioneer Co., Korea); subsequently, chromatograms were evaluated with CromasPro (version 1.5). The sequences of the *N* and *3'-UTR* genes were aligned with the corresponding region of the mentioned gene sequences from the GenBank. The genetic distances were calculated using the Kimura-two parameter model in the MEGA software (version 7). The phylogenetic tree was constructed using the Neighbor-Joining algorithm in the program using a consensus of 1,000 bootstrap replicates (Tamura et al., 2011).

RESULTS

According to the results, GCoV was detected in 8.99% of the samples collected from all parks, including 15% of chickens, 7.7% of quails, 4.2% of partridges, 27.3% of turkeys, and 8.8% of pheasants. Except for the samples recovered from partridges in two parks (located in the west and center of Tehran province), the virus was detected in every bird species in all parks. Moreover, the GCoV was not detected in the ducks, geese, and guinea fowls. The related data are shown in Table 1.

Table 1. The sample data and positive rate in cloacal swabs from birds parks, Tehran, 2015

Species		Number of Samples	Positive (%)
Chicken	(*Gallus gallus*)	20	15
Duck	(*Anas platyrhynchos*)	36	0
Goose	(*Anserini*)	24	0
Guinea fowl	(*Numididae*)	18	0
Partridge	(*Perdix perdix*)	24	4.2
Pheasant	(*Phasianus colchicus*)	34	8.8
Quail	(*Coturnix coturnix*)	78	7.7
Turkey	(*Meleagris gallopavo*)	33	27.3

Based on the partial nucleotide sequences of the *N* gene, the phylogenetic tree facilitated the categorization of the samples into two groups (Figure 1). The CoVs detected in turkeys, partridges, pheasants, and chickens were put in the same group with IR-Ur-1-09 (HQ607366) and IR-MNS7862-1 (HQ607365; i.e., two

Table 2. Primers that used for detection of avian gamma corona viruses.

Primer names	Gene	Primer sequences	Size band	Ref
UTR 41	UTR	ATG TCT ATC GCC AGG GAA AT GTC	266	20
UTR11		GCTCTAACTCTATAC TAG CCTA		
N103F	N	CCT GAT GGT AAT TTC CGT TGG G	357	21
N102R		ACG CCC ATC CTT AAT ACC TTC CTC		

Iranian IBV viruses; near *N* gene of TCoV and Mass IBV genotype). The GCoVs detected in quails were grouped with the CoV found in duck PL-MW284 2009, AvCoV bean goose PL-MW435 2009, and IS 117304 (near *N* gene of 793/B IBV genotype). Nucleotide sequences of *N* gene of turkeys' positive samples had the highest similarity to TCoV-540 (EU022525), H52, and M41, as well as to the Iranian IBV strains (i.e., IR-MNS7862-1 [HQ607365] and IR-Ur09 [HQ 607366]). Turkeys' samples had 98.61% and 96.09% similarities with Iranian IBVs.

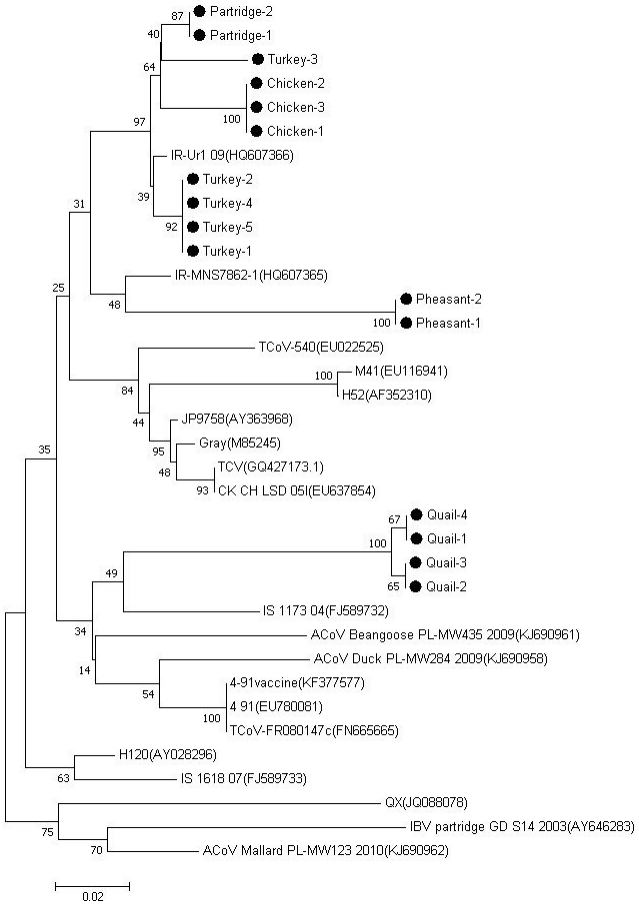

Figure 1. Phylogenetic tree based on Nucleocapsid of the Gammacorona viruses detected in bird parks, Iran, 2015. MEGA version 7, by the neighbor-joining method with 1000 bootstrap replicates (bootstrap values are shown on the tree) was used to construct the tree. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method.

Also, they showed 91.98%, 89.52%, and 89.41% similarities with TCoV-540 (EU022525), H52, and M41, respectively. The samples obtained from the chickens had a sequence identity of 96.91% and 94.49% with Iranian IBVs. Furthermore, these samples showed the sequence identity of 90.32%, 88.55%, and 88.02% with TCoV-540 (EU022525), H52, and M41, respectively. The CoVs detected in partridges had 98.61% and 96.10% sequence homology to Iranian IBVs. Regarding TCoV-540 (EU022525), H52, and M41, they demonstrated 92.38%, 90.82%, and 90.42% similarity, respectively. The two samples obtained from quails, which formed the next group, were 99.3% similar. The first sample had 87.89% and 87.60% sequence similarity with IS 117304 and AvCoV Duck PL-MW284 2009, respectively. Viruses recovered from pheasants had the sequence identities of 89.20%, 85.51%, and 85.08% with 4/91 (EU780081), H52, and M41, respectively. They had 79.66%, 88.59%, 89.24%, and 87.66% sequence similarity with viruses detected in quails, game birds, turkeys, and partridges, respectively. The UTR part of the genome was sequenced to confirm the CoV as well.

DISCUSSION

It is important to demonstrate the existence of CoVs in all kinds of animals, especially birds, since the presence of CoVs in birds, either with or without clinical signs, has been affirmed. The CoVs are the cause of important diseases, such as the Middle East respiratory syndrome and severe acute respiratory syndrome, and also avian infectious bronchitis in industrial birds. The bird parks may act as the sources of different viruses, as different hosts are gathered together, and viruses can spread rapidly. Therefore, it is a suitable place for viral recombination and interspecies transmission. Moreover, even virus transmission from birds to humans can occur in such places. Recently, the discovery of new viruses through next-generation sequencing technology, including DNA and RNA sequencing, has increased our knowledge about viral

diversity. The present study was the first attempt addressing the detection of CoV in the bird parks of Tehran province. Despite the presence of a large number of birds in both bird parks and live bird markets, no study has targeted the prevalence of GCoVs among these animals. In the current study, cloacal swabs were collected from three bird parks in Tehran. Negative and positive controls were considered in each step. Positive samples were sequenced for exact analysis. The virus was detected in pheasants, chickens, quails, partridges, and turkeys. The *N* gene-based phylogenetic tree facilitated the division of the samples into two groups. One of these groups included the CoVs detected in turkeys, partridges, chickens, and pheasants, and the other one entailed the CoVs found in quails. The *N* gene nucleotide sequences of three samples from turkeys had the highest similarity to Iranian IBV strains. Regarding the viruses obtained from game birds, they had the highest similarity to Iranian IBV strains, while partridges showed the highest similarity to Iranian IBVs. The great importance of GCoV infections in economy and health underscores the need for the implementation of epidemiological studies. The results revealed a high prevalence of AvCoVs among birds, except for chickens, indicating their potential role in spreading AvCoVs in different avifauna. The results of a study performed by Chu et al. demonstrated a high prevalence of novel AvCoV (12.5%) in aquatic wild birds (Chu et al., 2011). Furthermore, Durães-Carvalho et al. introduced birds as the potential new hosts distributing GCoVs (Durães-Carvalho et al., 2015). In a study conducted on Scandinavian waterfowl, the virus was mostly detected in the diving ducks, mainly Greater Scaup (*Aythya marila*; 51.5%) and the dabbling duck Mallard (*Anas platyrhynchos*; 19.2%). The prevalence rate of CoV was 18.7%, which is higher compared to the value (0-15%) reported by Wille et al. (2016). Domanska-Blicharz et al. (2014) characterized IB-like viruses in wild birds in Poland. Among the total CoV prevalence rate of 3.5%, detection rates in *Anseriformes* and *Charadriiformes* were 3.5% and 2.3%, respectively, while it was 17.6% in *Galliformes* (Domanska-Blicharz et al., 2014). Jiadong et al. suggested that a wild partridge CoV S14 which was isolated from the laryngotracheal swab may be originated from or related to nephrogenic-type and proventriculus-type IBV (Guihong et al., 2006). The results of the present study revealed that four avian species are susceptible to IBV-like CoVs. Analysis of the *RdRp* genes of GCoVs from ducks, sandpipers, and gulls showed that they are genetically close, and their overall *RdRp* gene sequence homology differs by < 7%. The results suggested the existence of genetically heterogeneous viruses in the same avian species, and also the presence of closely related viruses in different duck species. Moreover, it was revealed that four avian species are susceptible to IBV-like CoVs. Altogether it can be concluded that there are frequent intergenus and interspecies transmissions of GCoVs among avian species. What complicates the issue is the role of wild birds as major reservoirs of a wide range of GCoVs.

The importance of identifying the replication extent of AvCoV from one species and its pathogenic role in other bird species highlights the implementation of further studies to perform full genome analysis of AvCoVs. Moreover, additional studies are recommended to investigate coronavirus molecular epidemiology in different species of birds and full genome analysis of AvCoVs to provide precise insight into CoVs evolution.

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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