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Molecular Study of Gamma Coronaviruses in Birds from the Southern Caspian Sea Coast of Iran



Zoleikha Tatari¹, Arash Ghalyanchi Langeroudi^{1*}, Hossein Hosseini², Mohammad Malekan³, Zahra Ziafati Kafi¹, Rima Morshed⁴, Hamideh Najafi¹, Soroush Sasrudi¹, Naser Sadri¹, Nazanin Sarvian¹, Safoura Tatari¹

¹ Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

² Faculty of Veterinary Medicine, Karaj Branch, Islamic Azad University, Karaj, Alborz

³ Department of Veterinary Service, SAVAPARS (Ceva Sante Animale Exclusive Distributor in Iran), Tehran, Iran

⁴ Faculty of Encyclopedia, IHCS, Tehran, Iran

* Corresponding author email address: ghalyana@ut.ac.ir

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ABSTRACT

Gammacoronaviruses (γ -CoVs), including infectious bronchitis virus (IBV), cause economically important diseases in poultry and may act as reservoirs or bridges for viral dissemination. This study aimed to determine the frequency and genetic relationships of γ -CoVs in asymptomatic wild and free-ranging birds of northern Iran. From February to December 2020, 60 cloacal swabs were collected from 11 bird species ($n=3-10$ per species); RNA was extracted and a one-step RT-PCR targeting a 266-bp fragment of the 3' untranslated region (3' UTR) was performed, positive amplicons were Sanger-sequenced, sequences were aligned with reference γ -CoV sequences, and phylogenetic analysis was carried out. Six of 60 samples (10.0%) were RT-PCR positive—4/10 rural chickens (40.0%) and 2/10 white-headed ducks (20.0%)—while all other sampled species were negative. Sequence analysis of six isolates placed chicken-derived viruses in a cluster with known chicken IBV strains (one isolate clustering closely with the H120 vaccine sequence and two forming a separate subcluster), whereas the two duck-derived sequences were identical to each other and formed a distinct cluster with reference γ -CoV sequences from wild birds (e.g., duck, pigeon, and other avian hosts), indicating a shared lineage but not necessarily genetic identity with those reference strains. These findings indicate that asymptomatic free-ranging chickens and white-headed ducks in Golestan Province harbor γ -CoV RNA, suggesting local viral circulation and potential interactions at the wild-domestic interface.

Keywords: Gamma Coronavirus; Infectious Bronchitis Virus; Wild Birds; Rural Chickens; White headed Duck; Iran

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1 Introduction

Coronaviruses are enveloped positive-sense single-stranded RNA viruses with relatively large genomes (~27.5–28 kb) that encode nonstructural proteins from ORF1a/1ab and structural proteins at the 3' end, including spike (S), envelope (E), membrane (M), and nucleocapsid (N) (Abolnik, 2015; Milek & Blicharz Domańska, 2018; Su et al., 2016). Gammacoronaviruses (γ -CoVs) primarily infect birds and include the economically consequential infectious bronchitis virus (IBV), which causes respiratory and urogenital disease in poultry and substantial production losses (Jonassen et al., 2005; Rezaee et al., 2020). The S glycoprotein mediates receptor attachment and membrane fusion, while the N protein is involved in nucleocapsid formation and replication; E and M coordinate virion assembly and budding (Sheikh et al., 2020; Stern & Soften, 1982; Takiuchi et al., 2006). Four major coronavirus genera (Alpha, Beta, Delta, and Gamma) are recognized, with γ CoVs largely associated with avian hosts (Woo et al., 2012). Wild birds have been implicated as reservoirs for several avian viruses (Berg et al., 2001; Snoeck et al., 2013; Yaghoubi et al., 2019) and may contribute to the maintenance and geographic spread of γ -CoVs; however, the extent of their role in IBV ecology in Iran is incompletely characterized. This study aimed to detect γ -CoVs in

asymptomatic bird species on the Southern Caspian Sea Coast of Iran to characterize partial 3' UTR sequences to infer genetic relationships with known avian γ -CoVs.

2 Materials and Methods

2.1 Study design and sampling

Between February and December 2020, cloacal swabs were collected from 60 asymptomatic birds representing 11 species in the Southern Caspian Sea Coast of Iran (Table 1). This study was designed as an exploratory effort to detect γ CoV RNA in asymptomatic birds at the wild-domestic interface in Northern Iran. The sample size of 60 birds (3–10 per species) reflects logistical constraints, species availability during fieldwork, and the descriptive nature of the study rather than hypothesis-driven comparisons. Species were selected based on ecological relevance and potential contact with poultry. Species selection included free-ranging village chickens (*Gallus gallus*) and wild migratory and resident species with potential ecological contact with poultry. Samples were placed in viral transport medium, transported on dry ice, and stored at -70°C until processing. Animal handling and sampling procedures were approved by the Animal Ethics Committee of the University of Tehran (UTBE-1401). No invasive procedures or sacrifices were performed.

Table 1. Gamma Cor-V detected from sampled species in the Southern Caspian Sea Coast of Iran

No.	Common Name	Scientific Name	Order	No. of Samples	Positive Samples (%)
1	Rural Chicken	<i>Gallus gallus</i>	<i>Galliformes</i>	10	4 (40)
2	White-Headed Duck	<i>Oxyura leucocephala</i>	<i>Anseriformes</i>	10	2 (20)
3	Bar-Headed Goose	<i>Anser indicus</i>	<i>Anseriformes</i>	10	0
4	Honey Buzzard	<i>Pernis ptilorhynchus</i>	<i>Accipitridae</i>	5	0
5	Western Marsh Harrier	<i>Circus aeruginosus</i>	<i>Accipitridae</i>	5	0
6	Common Buzzard	<i>Buteo buteo</i>	<i>Accipitridae</i>	5	0
7	Egyptian Vulture	<i>Neophron percnopterus</i>	<i>Accipitridae</i>	3	0
8	Eurasian Eagle-Owl	<i>Bubo bubo</i>	<i>Strigiformes</i>	3	0
9	Common Kestrel	<i>Falco tinnunculus</i>	<i>Falconiformes</i>	3	0
10	Steppe Eagle	<i>Aquila nipalensis</i>	<i>Accipitridae</i>	3	0
11	Saker Falcon	<i>Falco cherrug</i>	<i>Falconiformes</i>	3	0
Total				60	6 (10)

2.2 RNA extraction and RT-PCR

Total RNA was extracted from swab medium using the High Pure RNA Extraction Kit (Roche, Germany) according to the manufacturer's instructions. One-step RT-PCR (Qiagen One-Step RT-PCR kit) targeted a 266-bp fragment of the 3' UTR using primers UTR41 (5'-ATGTCTATCGCCAGGGAAATGTC-3') and UTR11 (3'-

GCTCTAACTCTATACTAGCCTA-5') as previously described for broad γ -CoV detection (Cavanagh et al., 2002). Reaction composition and thermal profile followed the kit recommendations, with a final primer concentration of 400 nM; positive and negative controls were included in each run. Amplicons were visualized on agarose gel with EvaGreen loading dye.

2.3 Sequencing and phylogenetic analysis

Positive PCR products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen) and Sanger-sequenced (Bioneer Co., Korea). Raw sequences were quality-checked and edited in BioEdit v7.7.9, and compared to GenBank entries using BLAST. Multiple sequence alignments were generated in MEGA v7.0 (Kumar et al., 2018) using the ClustalW algorithm, and phylogenetic trees were constructed using the neighbor-joining method with p-distance as the substitution model (Saitou & Nei, 1987). Gaps and missing data were treated using pairwise deletion

to retain informative positions. Tree topology robustness was assessed with 1,000 bootstrap replicates.

The reference panel included representative gammacoronavirus sequences from GenBank spanning domestic poultry (e.g., IBV strains including H120), wild birds (e.g., duck, pigeon, and other avian hosts), and diverse geographic origins. The Turkey coronavirus (accession NC_010800) was used as the outgroup to root the tree. For clarity, the phylogenetic figure 1 includes host species, country of origin, and year of isolation for each sequence, highlighting clustering patterns and distinguishing wild-bird-associated and chicken-associated lineages.

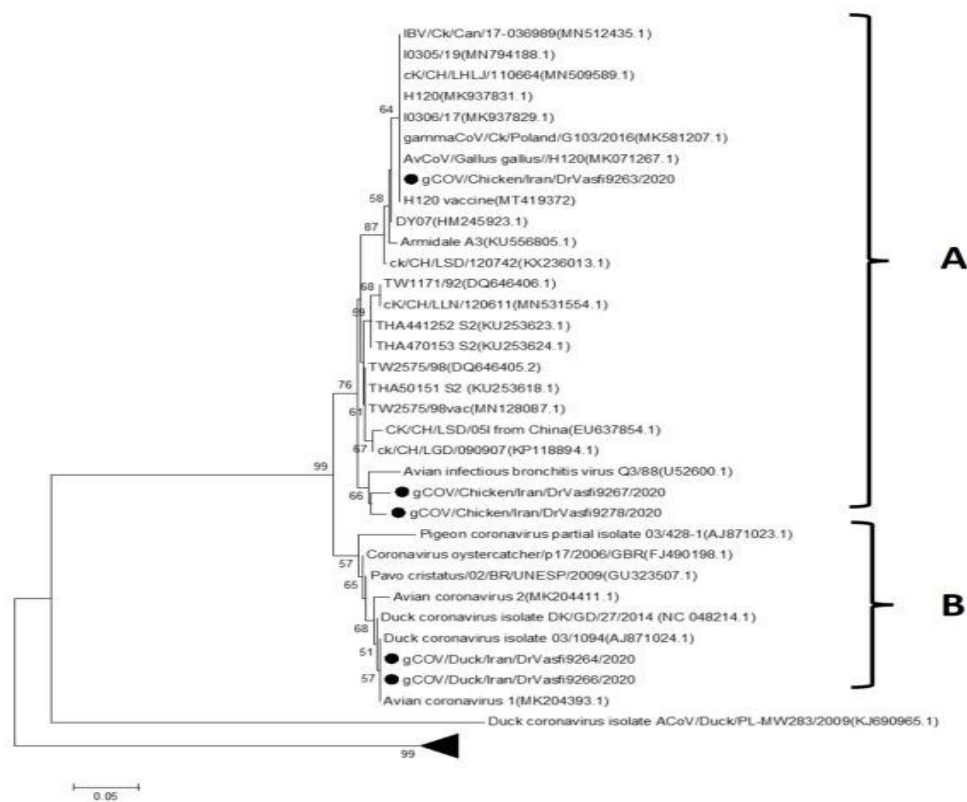


Figure 1. Phylogenetic tree of Gamma Coronaviruses isolates found in this study with other isolates. Phylogenetic analysis was performed with MEGA 7 software, using Maximum Likelihood method based on General Time Reversible model with 1000 bootstrap. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The analysis involved 48 nucleotide sequences. All positions with less than 95% site coverage were eliminated. Fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

2.4 Statistical considerations

The relative frequency was calculated as the proportion of positive samples per species. Exact binomial 95% confidence intervals (CIs) were computed to reflect

uncertainty due to small sample sizes: Overall frequency: 6/60 (10.0%; 95% CI: 3.8–20.5%); Chickens: 4/10 (40.0%; 95% CI: 12.2–73.8%); White-headed ducks: 2/10 (20.0%; 95% CI: 2.5–55.6%). Given the sample sizes per species (n=3–10), no formal hypothesis testing was performed;

results are presented descriptively with caution regarding limited statistical power for inter-species comparisons.

3 Results

Six of 60 cloacal swabs (10.0%) were RT-PCR positive for γ -CoV. By species: rural chickens 4/10 (40.0%), white-headed ducks 2/10 (20.0%); all sampled *Accipitriformes*, *Strigiformes*, and *Falconiformes* were negative (Table 1).

Sequencing was successful for five positive amplicons (three chicken-derived and two duck-derived) and one additional amplicon produced a sequence identical to one of

these (six isolates total, accession numbers MZ461452–MZ461456). Phylogenetic reconstruction based on the 3' UTR separated isolates into two clusters, consistent with prior reports that the 3' UTR can show host-associated groupings, though it is a conserved region with limited resolution for genotype assignment (Fig 1). Chicken isolates grouped with known IBV/chicken sequences; one chicken isolate clustered near H120 vaccine-related sequences, while two formed a separate subcluster. Duck isolates were identical to each other and clustered with duck/pigeon/wild-bird γ -CoV references, such as Duck coronavirus 03/1094 and Avian coronavirus sequences. The pairwise nucleotide identity matrix is summarized in Table 2.

Table 2. Percent of similarity between 3' untranslated region (UTR) of gamma-CorV strains of the current study and other gamma-CorVs acquired from GenBank.

		1	2	3	4	5	6	7	8	9	10	11	12
1	gCOV/Chicken/Iran/DrVasfi9267/2020												
2	gCOV/Chicken/Iran/DrVasfi9278/2020	97.18											
3	gCOV/Chicken/Iran/DrVasfi9263/2020	93.79	94.35										
4	gCOV/Duck/Iran/DrVasfi9264/2020	92.09	93.79	92.66									
5	gCOV/Duck/Iran/DrVasfi9266/2020	92.09	93.79	92.66	100.00								
6	IBV Q3/88 (U52600.1)	94.92	95.48	94.35	91.53	91.53							
7	AvCoV/Gallus/gallus/H120(MK071267.1)	93.79	94.35	100.00	92.66	92.66	94.35						
8	H120 vaccine (MT419372)	93.79	94.35	100.00	92.66	92.66	94.35	100.00					
9	Duck coronavirus 03/1094 (AJ871024.1)	92.09	93.79	92.66	100.00	100.00	91.53	92.66	92.66				
10	Avian coronavirus 1(MK204393.1)	92.09	93.79	92.66	100.00	100.00	91.53	92.66	92.66	100.00			
11	Avian coronavirus 2 (MK204411.1)	91.53	93.22	92.09	98.87	98.87	90.96	92.09	92.09	98.87	98.87		
12	DK/GD/27/2014 (NC_048214.1)	91.53	93.22	92.09	99.44	99.44	92.09	92.09	92.09	99.44	99.44	98.31	

4 Discussion

Detection of γ -CoV RNA in asymptomatic free-ranging chickens and white-headed ducks in Northern Iran aligns with multiple reports of γ -CoV circulation among *Anseriformes* and *Galliformes* in diverse regions and supports the concept that wild aquatic birds can carry γ -CoVs without overt disease (Rahman et al., 2021).

Phylogenetic placement of chicken-derived viruses with IBV lineages, including an H120-related sequence, may reflect vaccine-derived strains circulating in village flocks, vaccine virus shedding, or sequence conservation in the 3' UTR; distinguishing these possibilities requires higher-resolution sequencing, such as S1 or whole-genome analysis. Duck-derived viruses clustered with wild-bird γ -CoV lineages, supporting a distinct wild-bird-associated lineage in the region and consistent with previous detections of diverse γ -CoVs across wild *Anseriformes* and other

avifauna (De Sales Lima et al., 2015; Domanska-Blicharz et al., 2014; Rahman et al., 2021; Rohaim et al., 2021).

The overall relative frequency (10%) is comparable to several surveillance studies of wild aquatic birds that reported variable prevalence depending on sampling design and genomic target. Rahman et al. reviewed studies on Cor-Vs across different countries and reported that the prevalence of infection among *Anseriformes* and *Galliformes* was 75% and 12.5%, respectively (Rahman et al., 2021). Infection of Cor-Vs in mallard ducks (*Anas platyrhynchos*) and graylag goose (*Anser anser*) had been documented in Norway and Sweden based on order. In Norway, the bird populations of *Anser anser*, wild pigeon (*Columba livia*), and mallard (*Anas platyrhynchos*) were screened. In sampled birds, 40 of 163 were CoV-positive in the graylag goose, whereas 2 of 100 sampled pigeons and 1 of 5 sampled mallards tested positive (Jonassen et al., 2005). The prevalence of CoVs among wild waterbirds in Sweden was 18.7%, reported by Wille et al (Wille et al., 2016). Chu et al. surveyed in China and found that 12% of even

asymptomatic wild aquatic birds were positive for CoVs, and gamma CoVs were predominantly identified in *Anseriformes* (Chu et al., 2011).

Migratory birds, especially *Anseriformes*, are an excellent reservoir of various pathogens and serve as reactors due to their high species diversity and ecological habits, such as gathering during feeding (Miłek & Blicharz Domańska, 2018). Therefore, they appear to play a significant role in disseminating and surviving the Cor-Vs within a geographical region. Paim *et al.* suggested that wild birds without clinical signs could be a reservoir for Cor-Vs (Paim et al., 2019). An investigation in Finland revealed that a gamma Cor-V identified in ducks was closely related to the Cor-V identified in ducks from Siberia and China. This finding suggests that highly mobile hosts may be able to move the virus across large distances (Hepojoki et al., 2017). In 1997, Traavik *et al.* detected a coronavirus-like virus in ticks collected from seabirds in Norway (Traavik et al., 1977). Also, gamma Cor-Vs have been isolated from teal (*Anas*), peafowl (*Pavo cristatus*), racing pigeon (*Columba livia domestica*), as well as Scaly-breasted munia (*Lonchura punctulata*), grey-backed thrush (*Turdus hortulorum*), mallard (*Anas platyrhynchos*), graylag goose (*Anser anser*), blackbirds (*Turdus merula*), white-rumped munia (*Lonchura striata*), Chinese Bulbul (*Pycnonotus sinensis*), and rock dove (*Columba livia*) (Jonassen et al., 2005; Liu et al., 2005; Woo et al., 2009). Domestic and wild *Anseiformes* could serve as a mixing vessel for different Cor-Vs. Due to domestic ducks' high susceptibility to Cor-Vs of wild *Anseriformes*, these domestic ducks could play a role in cross-species transmission of the virus to nearby poultry farms (Pauly et al., 2019).

Asymptomatic free-ranging chickens and white-headed ducks in Northern Iran harbor γ -CoV RNA. Partial 3' UTR phylogeny indicates chicken isolates grouped with known IBV/chicken sequences; one isolate showed close similarity to H120 vaccine-related sequences, suggesting compatibility with a vaccinal or vaccinal-like origin. However, due to the conserved nature of the 3' UTR region, definitive differentiation between vaccine-derived and field strains is not possible without higher-resolution sequencing, such as S1 or whole-genome analysis. These results support expanded surveillance, higher-resolution sequencing, and integrated ecological studies to clarify the role of wild birds in local γ -CoV circulation and to guide poultry disease control strategies.

5 Limitations

Study limitations include the use of a short conserved 3' UTR fragment that limits genotype discrimination and recombination detection, small per-species sample sizes limiting statistical power, and detection of RNA only, which does not prove infectious virus or active replication. Future work should include targeted S1 and whole-genome sequencing, virus isolation and phenotypic characterization, longitudinal sampling at wild-domestic interfaces, and integration of ecological and farm-practice data to model spillover risk and inform biosecurity measures.

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AI Use Statement

Artificial intelligence tools were not used for data analysis or interpretation. AI assistance was limited to language editing and formatting during manuscript preparation.

Conflict of Interest

We declare that no conflict of interest.

Author Contributions

The study was conceptualized by A.GH and H.H. Sampling and fieldwork were carried out by N.S, Z.T, M.M, S.S, N.S, and A.A. H.N, Z.Z, O.E, and S.T conducted the laboratory analyses. Data analysis and interpretation were performed by H.H, A.GH, and R.M. The manuscript was drafted by A.GH and R.M, while overall supervision and project administration were provided by A.GH.

Data Availability Statement

Sequences generated in this study are deposited in GenBank (MZ461452–MZ461456). Other datasets are available from the corresponding author upon reasonable request.

Ethical Considerations

Sampling and procedures were approved by the Animal Ethics Committee of the University of Tehran (Approval

Code: UTBE-1401). No birds were sacrificed; handling followed approved protocols.

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