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# MOLECULAR AND CELLULAR BIOLOGY

## Gamma and Deltacoronaviruses in quail and pheasants from Northern Italy<sup>1</sup>

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**ABSTRACT** In view of the restricted knowledge on the diversity of coronaviruses in poultry other than chicken, this study aimed to investigate the genetic diversity of coronaviruses in quail, pheasant, and partridge from two regions of Northern Italy. To this end, pools of tracheal and cloacal swabs from European quail (*Coturnix Coturnix*) and intestinal tract from pheasants (*Phasianus Colchicus*) and partridge (*Perdix Perdix*) flocks, with or without enteric signs, were collected during 2015. Avian coronavirus (*Gammacoronavirus*) was detected in quail not vaccinated against Infectious

Bronchitis Virus (IBV) and in pheasants vaccinated with an IBV Massachusetts serotype. Based on DNA sequences for the gene encoding the S protein, the avian coronaviruses detected in the quail and pheasant are related to the IBV 793B and Massachusetts types, respectively. However, RNA-dependent RNA polymerase (*RdRp*) analyses showed the susceptibility of quail also to Deltacoronaviruses, suggesting that quail and pheasant avian coronaviruses share spike genes identical to chicken IBV spike genes and quail might host *Deltacoronavirus*.

**Key words:** *Gammacoronavirus*, *Deltacoronavirus*, quail, pheasant, infectious bronchitis virus

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## INTRODUCTION

Coronaviruses (**CoV**), positive-sense single stranded RNA viruses (*Nidovirales: Coronaviridae: Coronavirinae*), are currently classified in four genera. *Alpha-coronavirus* ( **$\alpha$ -CoV**) and *Betacoronavirus* ( **$\beta$ -CoV**) infect many mammalian species ranging from bats to humans (Chan, et al., 2013) whereas *Gammacoronavirus* ( **$\gamma$ -CoV**) and *Deltacoronavirus* ( **$\delta$ -CoV**) are largely established in birds and to a slightly degree in mammals (Woo et al., 2010; Woo et al., 2012; De Groot et al., 2012; Fehr and Perlman, 2015).

*Avian coronavirus* (Avian infectious bronchitis virus, Infectious Bronchitis Virus [**IBV**] in chickens), the causative agent of avian infectious bronchitis (**IB**), is a *Gammacoronavirus* that can replicate on epithe-

lial tissues, affecting respiratory, reproductive, urinary, and enteric tracts (Cavanagh, 2003; Cavanagh, 2005; Villarreal et al., 2007). IBV and IBV-like strains can be detected in both gallinaceous and non-gallinaceous birds, such as geese, ducks, pigeons, pheasants (Cavanagh et al., 2002), and quail (Circella et al., 2007; Torres et al., 2013), asymptotically in some cases (Cavanagh, 2005). This might suggest that these species would act as vectors spreading IBV strains over the world (De Wit et al., 2011).

Other coronaviruses, classified into the *Deltacoronavirus* genus (De Groot et al., 2012), were described by Woo et al. (2009), Chu et al. (2011) and Durães-Carvalho et al. (2015) in wild birds.

In the view of the restricted knowledge on coronaviruses in poultry other than chickens, this study aimed to investigate the genetic diversity of coronaviruses in farmed quail, pheasants and gray partridges from Northern Italy.

## MATERIAL AND METHODS

### Farms

The survey was performed on 10 farms including 17 quail, 8 pheasant, and 2 gray partridge flocks in 2015.

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<sup>1</sup>The nucleotide sequence data reported in this paper have been submitted to GenBank Submission (8600 Rockville Pike, Bethesda MD, 20894 USA) nucleotide sequence database and have been assigned the accession number KX077959-KX077975.

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**Table 1.** Identification and characteristics of the Italian farms studied during April to July of 2015, regarding type of bird (quail, pheasant, and gray partridge), time of sampling (beginning, middle, end, or complete production cycle), type of sample, and IB vaccination programs applied.

Farm ID	Bird type	Sampling time	Type of sample	IB vaccination program
F1	Quail/Broiler	CPC <sup>#</sup>	CS/TS	Broilers vaccinated at hatchery = Mass and 793B
F2	Quail	CPC	CS/TS	Non vaccinated
F3	Quail	Beginning/middle	Intestine	Non vaccinated
F4	Quail	CPC	CS/TS	Non vaccinated
F5	Quail	CPC	Intestine	Quail breeders = IBV Mass
F6	Pheasant	CPC	Intestine	One day old = IBV Mass
F7	Pheasant	Middle	Intestine	Non vaccinated
F8	Pheasant	Middle	Intestine	Non vaccinated
F9	Pheasant	Middle/end	Intestine	Non vaccinated
F10	Partridge	End	Intestine	Non vaccinated

<sup>#</sup>= farm 1 was sampled in three following production cycles.

IB = infectious bronchitis.

CPC = Complete production cycle (beginning, middle and end).

CS = Cloacal swab.

TS = Tracheal swab.

Mass = Massachusetts serotype.

Characteristics of flocks, vaccination programs applied, and sampling details are summarized in Table 1. Farms included in the study were all located in Northern Italy, in particular in the Veneto and Emilia Romagna regions that are considered densely populated poultry areas.

For each flock, data regarding the occurrence of respiratory or enteric signs ongoing or experienced were recorded.

### Sample Collection

Cloacal and tracheal dry swabs (10 birds/flock) or the intestinal tract (5 samples/flock) from necropsied birds, were collected for molecular investigation. The swabs for the molecular testing were dried at room temperature for approximately 15 minutes.

All samples were stored at  $-80^{\circ}\text{C}$  until processing as a pool according to the sampling (dry swabs or intestinal tract).

### RNA Extraction

Pools of tracheal and cloacal swabs were eluted in 2 mL of sterile PBS and pools of intestinal tissues and contents were prepared as a 50% (v/v) suspension in PBS and clarified at  $12,000 \times g$  for 15 min at  $10^{\circ}\text{C}$ . Total RNA was extracted from 200  $\mu\text{L}$  of the supernatants and from elutions using High Pure RNA Isolation kit (Roche Diagnostics Italy) according to the manufacturer's instructions.

### One Step Nested RT-PCR for Avian Coronavirus Screening

Each sample was screened for *Avian coronavirus* as described by Cavanagh et al. (2002) targeting the 3'-untranslated region (UTR) using SuperScript III RT/

Platinum Taq Mix (Invitrogen, Carlsbad, CA) as per manufacturer's instructions.

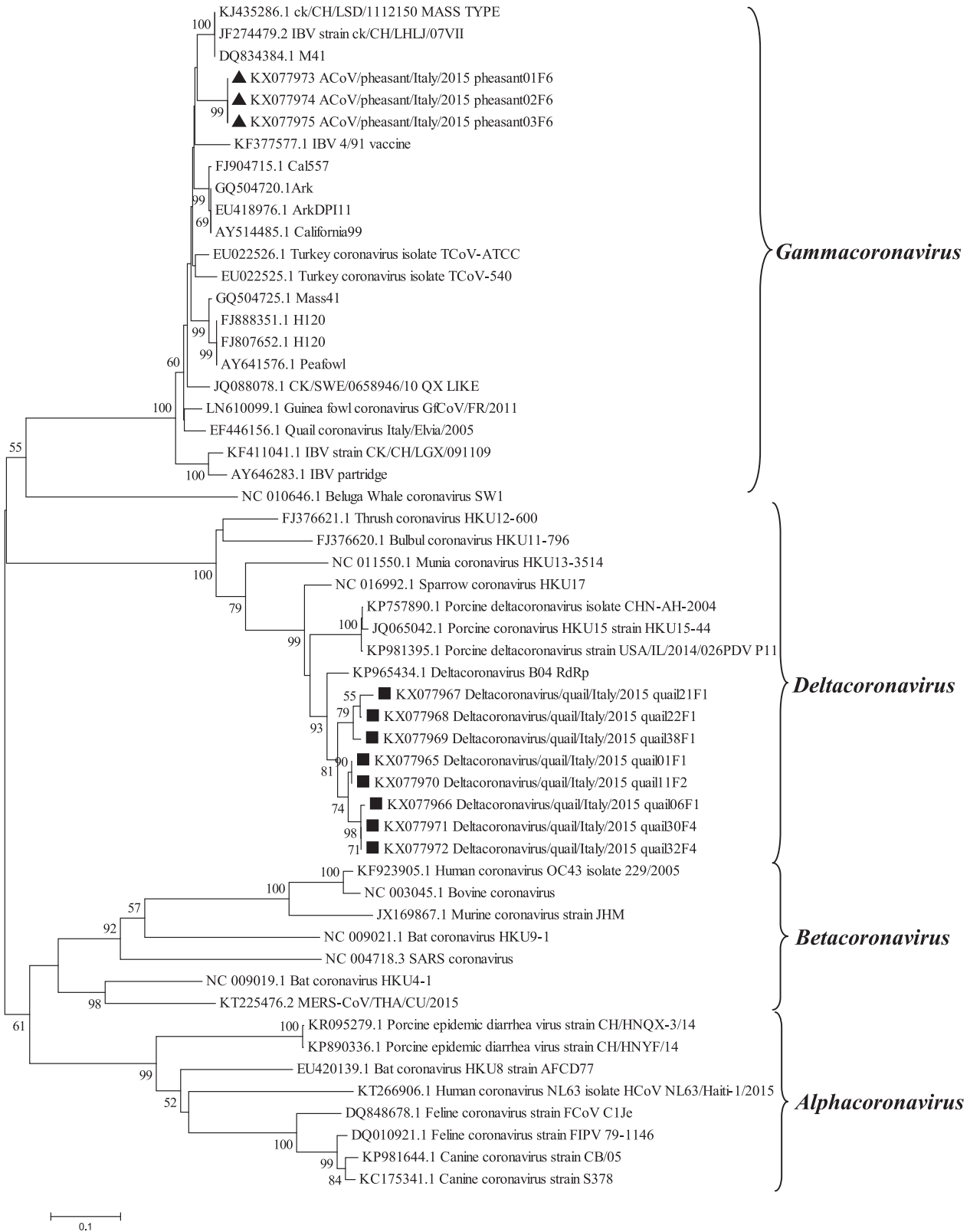
### RNA-dependent RNA Polymerase (RdRp) and Spike Gene Analysis

3'UTR- *Avian coronavirus* positive samples were then tested using the following protocols: 1) a pan-coronavirus RT-PCR targeting the RNA-dependent RNA polymerase (*RdRp*) gene as described by Chu et al. (2011) (440 base of pairs [bp] amplicon); 2) a S-gene targeted multiplex RT-PCR for 793B, D274 and Massachusetts types (Cavanagh et al., 1999) and 3) finally a generic S gene RT-PCR described by Worthington et al. (2008) (393 bp amplicon). All RT-PCR were performed using SuperScript III RT/ Platinum Taq Mix (Invitrogen, Carlsbad, CA) as per manufacturer's instructions.

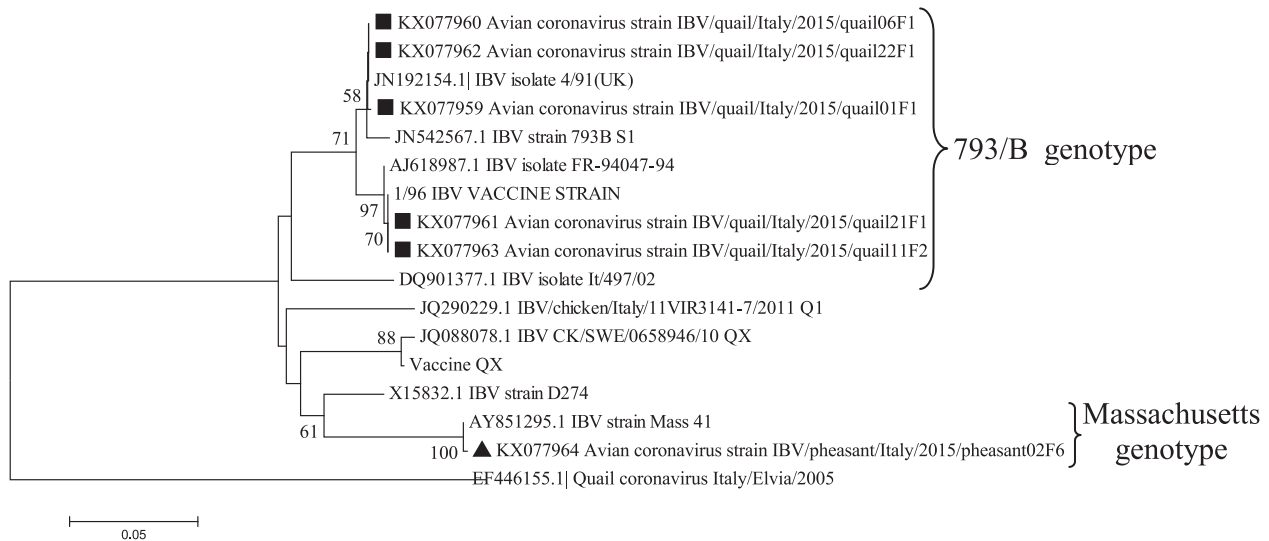
All RT-PCR amplicons were analyzed by electrophoresis in 2% (w/v) agarose stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA) and visualized by ultraviolet transillumination.

DNA sequencing was performed at MacroGen (MacroGen Europe, Amsterdam, Netherlands). All chromatograms were manually checked with Finch TV program. 1.4.0 (2004–2006 Geospiza Inc) and submitted to quality evaluation by Phil's Read Editor (PHRED) online application <http://asparagin.cenargen.embrapa.br/phph/> with a base-quality cut-off of 20. The final sequence of each sample was obtained with the Consensus application included in BioEdit 7.0.9.0 software (Tom Hall © 1997–2007) and aligned with homologous sequences retrieved from GenBank (see accession numbers in Figures 1 and 2) with Clustal/W multiple alignment using the same software.

Nucleotide (Neighbor-joining, Maximum Composite Likelihood model, 1,000 bootstrap replications) trees for S and *RdRp* genes were built using MEGA 5.2.1 (Tamura et al., 2011).



**Figure 1.** Phylogenetic tree based on the alignments of nucleotide sequences of *RdRp* gene (nt position 16,179 to 16,543) of samples sequenced in this study (square for quail and triangle for pheasant) and the prototype serotypes/genotypes (with Genbank accessing numbers) of the genera *Alpha*, *Beta*, *Gamma*, and *Deltacoronavirus*. The numbers above each node represent the bootstrap values for 1,000 replicates (only values greater than 50% are shown). The bar represents the number of nucleotide substitutions per site.



**Figure 2.** Phylogenetic tree based on the alignments of nucleotide sequences of partial S gene (nt position 726 to 1,073) of samples sequenced in this study (square for quail and triangle for pheasant) and the prototype serotypes/genotypes (with Genbank accession numbers). The numbers above each node represent the bootstrap values for 1,000 replicates (only values greater than 50% are shown). The bar represents the number of nucleotide substitutions per site.

## RESULTS

### Avian Coronavirus Screening

Fifteen flocks (9 of quail and 6 of pheasant) from 7 farms (F1, F2, F4, F5, F6, F7, and F8) were positive for *Avian coronavirus* UTR targeted RT-PCR. Gray partridge samples were negative.

IBVs detected by the multiplex typing PCR belonged to the 793B and Massachusetts genotypes in quail and pheasants, respectively. Strains amplified in *RdRp* and S genes are reported in Table 2.

The phylogenetic tree (Figure 1) for the *RdRp* gene indicates that all quail strains clustered in the  $\delta$ -CoV genus. Mean *RdRp* nucleotide identities of the quail strains with *Deltacoronavirus* B04 genotype (Genbank

KP965434) (Durães-Carvalho et al., 2015) and Porcine *Deltacoronavirus*, was 92.5% and 86%, respectively. Moreover, three subclusters were observed, with a mean nucleotide identity of 95.89%, suggesting the existence of different viral populations.

Strains detected in pheasants clustered in the *Gammacoronavirus* genus (Figure 1), with 100% identity amongst each other.

The phylogenetic tree based on the alignments of nucleotide sequences of partial S1 (Figure 2) confirmed the results of the multiplex RT-PCRs. All quail strains analyzed belonged to the 793B IBV genotype (mean nucleotide identity of 97%). Two strains (accession numbers KX077963 and KX077961) clustered with a commercial IBV vaccine based on 793B genotype widely used in Italy (mean nucleotide identity of 99.4%). The

**Table 2.** Strains included in the phylogenetic analysis of the partial RNA-dependent RNA polymerase (*RdRp*) and S genes according to species (quail or pheasant), clinical signs at the sampling time and sample.

Strain	Host	Signs	Sample	Gene	GenBank accession number
Quail01/CS/F1	Quail	N/S	Cloacal swab	<i>RDRP</i>	KX077965
Quail01/CS/F1	Quail	N/S	Cloacal swab	S	KX077959
Quail06/CS/F1	Quail	N/S	Cloacal swab	<i>RDRP</i>	KX077966
Quail06/CS/F1	Quail	N/S	Cloacal swab	S	KX077960
Quail21/CS/F1	Quail	N/S	Cloacal swab	<i>RDRP</i>	KX077967
Quail21/CS/F1	Quail	N/S	Cloacal swab	S	KX077961
Quail22/TS/F1	Quail	N/S	Tracheal swab	<i>RDRP</i>	KX077968
Quail22/TS/F1	Quail	N/S	Tracheal swab	S	KX077962
Quail38/CS/F1	Quail	Ent	Cloacal swab	<i>RDRP</i>	KX077969
Quail11/TS/F2	Quail	Ent	Tracheal swab	<i>RDRP</i>	KX077969
Quail11/TS/F2	Quail	Ent	Tracheal swab	S	KX077963
Quail30/CS/F4	Quail	Ent	Cloacal swab	<i>RDRP</i>	KX077971
Quail32/CS/F4	Quail	Ent	Cloacal swab	<i>RDRP</i>	KX077972
Pheasant01/INT/F6	Pheasant	Ent	Intestine	<i>RDRP</i>	KX077973
Pheasant02/INT/F6	Pheasant	Ent	Intestine	<i>RDRP</i>	KX077974
Pheasant03/INT/F6	Pheasant	Ent	Intestine	<i>RDRP</i>	KX077975
Pheasant02/INT/F6	Pheasant	Ent	Intestine	S	KX077964

Ent = Enteric.  
N/S = Not signs.

strains detected in pheasants clustered all with Massachusetts IBV genotype with 99% nucleotide identity.

## DISCUSSION

The presence and molecular diversity of coronaviruses in quail, pheasants, and partridge from Northern Italy was investigated, allowing the detection of *Avian coronavirus* related in the spike gene to 793B and Massachusetts IBV genotypes in quail and pheasants, respectively.

The phylogenetic analysis of the S gene suggested circulation in quails of 793B IBV genotype. This genotype, after its first appearance in the 1990s (Gough et al., 1992), became one of the most widespread genotypes in Europe (Worthington et al., 2008); for its control homologous live vaccination is widely used.

Vaccine origin of IBV detections in quail could not be excluded because molecular tools currently available are unable to unequivocally distinguish between field and vaccine viruses. Furthermore, recent epidemiological study on broilers strongly suggests that 793B detected in Italy derived from the 793B vaccines in use (Franzo et al., 2014).

High homology of a 793B genotype, detected in quail, with the identical serotype used in the vaccine applied to a broilers flock housed in the same farm strongly supports this hypothesis.

Pheasants IBV spike detection clustered with Massachusetts genotype. Considering that the pheasants had been vaccinated with a Massachusetts live vaccine, this also suggests a vaccine origin for this detection, as IBV vaccines might be detected in vaccinated birds for weeks after vaccination (Cavanagh et al., 1999). The sequence of *RdRp* of the same sample clustered within Gammacoronaviruses.

Surprisingly the *RdRp* analysis showed that quail detections clustered with  $\delta$ -CoV and not with  $\gamma$ -CoV as expected. The previously reported Quail coronavirus (QCoV) (Circella et al., 2007) based on the *RdRp* gene showed that QCoV was grouped along with  $\gamma$ -CoV and the S1 subunit of the S gene, showed 16 to 18% amino acid identity with IBV and 79 to 81% identity with TCoV (turkey coronavirus) strain, suggesting that specifically QCoV was not an IBV variant.

Taking into account the discrepant taxonomy of the quail coronaviruses detected herein regarding the *RdRp* and the spike genes (*Deltacoronavirus* and *Gammacoronavirus*, respectively) both a *Gamma-Deltacoronavirus* strain co-circulation or occurrence of a recombination event might be hypothesized. Nonetheless, recombination between-genera in coronaviruses, though initially considered but latter disregarded as a source of the SARS coronavirus, seems to be unlikely in nature (Thor et al., 2011, Woo et al., 2010).

Further efforts on full-genome sequencing using next generation sequencing of the quail coronaviruses de-

tected herein will better clarify the characteristics and origin of the detected strains.

Isolation and inoculation of quails in experimental conditions will confirm the susceptibility of this avian species to  $\delta$ -CoV.

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