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Source: Avian Diseases, 57(2) : 295-299

Published By: American Association of Avian Pathologists

URL: https://doi.org/10.1637/10412-100412-Reg.1

AVIAN DISEASES 57:295–299, 2013

Research Note—

An *Avian Coronavirus* in Quail with Respiratory and Reproductive Signs

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Received 24 September 2012; Accepted 23 January 2013; Published ahead of print 25 January 2013

SUMMARY. An *Avian coronavirus* was detected in pools of lungs, tracheas, female reproductive tracts, kidneys, and enteric contents from quail (*Coturnix coturnix japonica*) and laying hen flocks, with and without infectious bronchitis (IB)–like signs, cohoused in farms located in two states of southeastern Brazil during 2009–2010. Although *Avian metapneumovirus* subtype B was found in two layers samples, *Newcastle disease virus* was not found in quail or in hens. Based on DNA sequences for the 3′-untranslated region and the gene encoding the RNA-dependent RNA polymerase, this avian coronaviruses in quail is an IB virus–like gammacoronavirus.

RESUMEN. *Nota de Investigación*—Un coronavirus aviar en codornices con signos respiratorios y reproductivos.
Se detectó un coronavirus aviar en muestras agrupadas de pulmones, tráqueas, tractos reproductivos femeninos, riñones y contenidos intestinales de parvadas de codornices (*Coturnix coturnix japónica*) y de gallinas ponedoras, con la presencia o ausencia de signos clínicos sugestivos de la bronquitis infecciosa. Estas parvadas estaban alojadas conjuntamente en granjas ubicadas en dos estados del sureste de Brasil durante el período entre los años 2009 al 2010. Aunque se encontró metapneumovirus aviar subtipo B en dos muestras de ponedoras, no se encontró al virus de la enfermedad de Newcastle en las codornices ni en las gallinas. Con base en las secuencias de ADN de la región 3′no traducida y del gene que codifica a la polimerasa de ARN dependiente de ARN, este coronavirus aviar de las codornices es un virus cercano a los gammacoronavirus.

Key words: *Avian coronavirus*, infectious bronchitis virus, quail, laying hens

Abbreviations: aMPV = *Avian metapneumovirus*; DEPC = diethyl pyrocarbonate; IB = infectious bronchitis; IBV = infectious bronchitis virus; NDV = *Newcastle disease virus*; nt = nucleotide(s); *RdRp* = RNA-dependent RNA polymerase gene; RT = reverse transcription; S-gene = spike gene; UTR = untranslated region

Avian infectious bronchitis virus (IBV), the causative agent of avian infectious bronchitis (IB), is a *Gammacoronavirus* that replicates in lungs, tracheas, kidneys, guts, and reproductive tracts of breeders, broilers, and layers, leading to massive economic losses due to delayed growth, egg production drop, eggshell malformations, and infertility (3,8,13,24,27). Other respiratory pathogens, such as *Newcastle disease virus* (NDV) and *Avian metapneumovirus* (aMPV), can interact with IBV, increasing the severity and the course of the disease (20). Gammacoronaviruses also were detected in asymptomatic birds, including peafowl (*Pavo cristatus*), teals (*Anas* spp.) (19), feral pigeons (*Columba livia*) (17,19), graylag geese (*Anser anser*), and mallard ducks (*Anas platyrhynchos*) (19) and in several species of wild birds in captivity (2).

In quail (*Coturnix coturnix japonica*), the first report of coronavirus-like viruses was made in 1983 by Pascucci *et al*. (23), with strains isolated from birds with a respiratory syndrome. Two years later, Pascucci *et al*. (22) showed by virus neutralization and immunoelectroscopy that one of the strains was not related to IBV or mammalian coronaviruses. In 2005, a coronavirus was associated with an enteric syndrome in quail and named quail coronavirus (9).

IBV has already been isolated from healthy and nonvaccinated quail, showing a low antigenic identity with IBV serotypes Massachusetts, Connecticut, and D207 in Brazil (13). Despite these few reports, there is still little information on the molecular diversity of coronaviruses in quail and on the role of this host in the epidemiology of avian coronaviruses. This study thus investigated the occurrence and molecular diversity of *Avian coronavirus* in quail and laying hens raised on the same farms and determined the role of quail in the epidemiology of avian IB.

MATERIALS AND METHODS

Source of viruses. Quail and laying hens raised together on farms in the states of São Paulo and Espírito Santo, municipalities of Bastos and Santa Maria de Jetibá, respectively, in southeastern Brazil, were sampled in 2009 and 2010. These farms had a history of chickens and quail with respiratory signs such as nasal discharge, gasping, watery eyes, and conjunctivitis and reproductive signs such as egg production drop, eggs with thin-walled and misshapen shells with loss of pigmentation, and ''false layers'' (birds who ovulate without egg production). At necropsy, there was severe oviduct edema in most of the chickens and quail. No additional specific gross lesions were observed.

Samples were collected from five birds (quail or chickens) per house, as pools of female reproductive tracts, lungs, kidneys, and tracheas and complete enteric contents from quail. Birds were sampled in six farms (from 18 quail and 12 chicken flocks) resulting in a total of 90 samples from quail and 48 samples from chickens from both healthy and symptomatic birds.

In all studied farms, chickens had been vaccinated against IBV by using attenuated Massachusetts vaccines plus inactivated Massachusetts vaccines, against aMPV with subtypes A or B and against NDV with a lentogenic vaccine. Quail were not vaccinated against IBV and aMPV, but they were vaccinated with the lentogenic NDV.

IBV, aMPV, and NDV reference strains. The IBV Massachusetts strain vaccine (NOBILIS™ IB Ma5; MSD Animal Health, Boxmeer,

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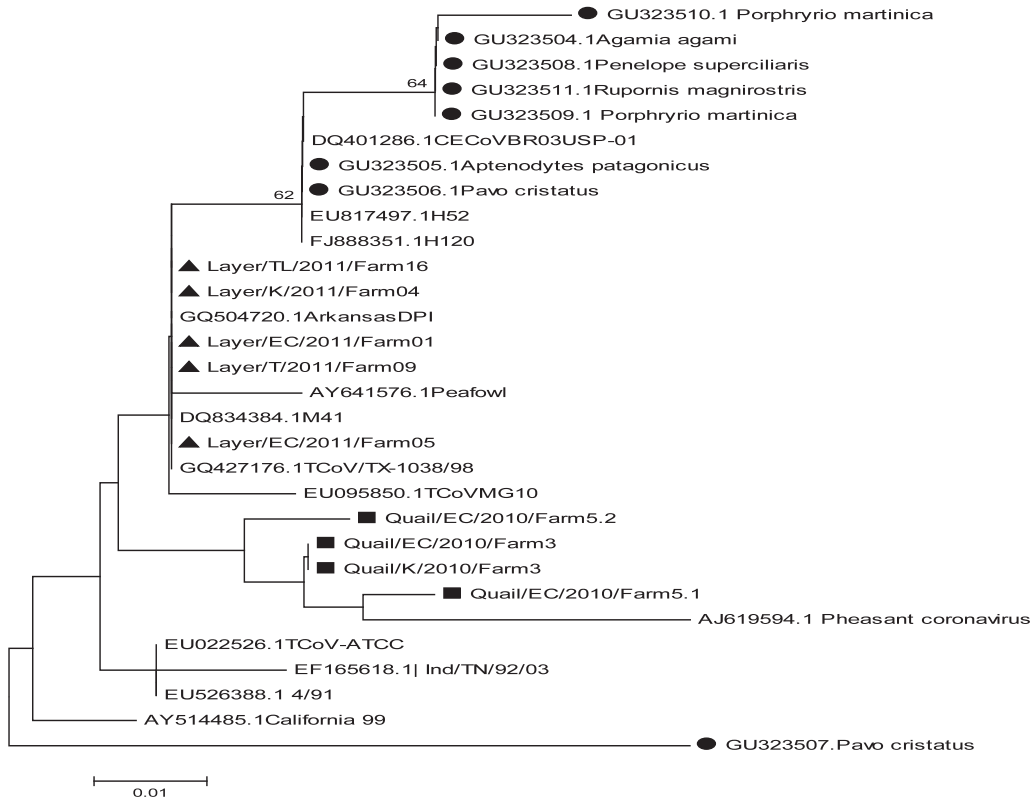

Fig. 1. Phylogenetic distance tree with the neighbor-joining algorithm for nucleotides of the 3′-UTR (nt 27,342–27,520), showing the classic serotypes/genotypes (with GenBank accession numbers) and strains included in this study (squares for quail, triangles for laying hens, and circles for Brazilian wild bird *Gammacoronavirus* strains). The numbers above each node represent the bootstrap values for 1000 replicates (only values >50% are shown). The bar represents the number of nt substitutions per site.

The Netherlands), aMPV strain vaccine RTV 8544 (NOBILIS™ RTV 8544; MSD Animal Health) and NDV lentogenic strain vaccine (ND LA SOTAL NOBILIS™; MSD Animal Health) were used as positive controls for reverse transcription (RT)-PCR. As negative controls, ultrapure water treated with 0.1% diethyl pyrocarbonate (DEPC) was included.

Extraction of RNA. Pools of organs and enteric contents were prepared as 50% (v/v) suspensions in DEPC-treated water and submitted to three freeze-thaw cycles in liquid nitrogen and 56 C, and then they were clarified at 5000 × *g* for 15 min at 4 C. Total RNA was extracted from the supernatants with TRIzol Reagent™ (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions.

Synthesis of cDNA. We denatured 3.5 µl of RNA of each RNA at 94 C for 5 min and then reverse transcribed the samples with Random Primers™ and M-MLV Reverse Transcriptase™ (Applied Biosystems) as per the manufacturer's instructions.

IBV, aMPV, and NDV screening. Each sample was screened for IBV, aMPV, and NDV as described by Cavanagh *et al.* (6,7) and Tiwari *et al.* (26), targeting the 3′-untranslated region (UTR), G gene, and F gene, respectively, by using Platinum® Taq DNA Polymerase (Applied Biosystems) per the manufacturer's instructions, with amplicons of 179, 268 or 361 (aMPV types A or B), and 255 bp, respectively.

RNA-dependent RNA polymerase (*RdRp*) gene partial amplification and IBV genotyping. The 3′-UTR–positive samples were then tested by an RT-PCR targeting the *RdRp* gene as described by Escutenaire *et al.* (15) and by a typing spike (S)-gene–targeted multiplex RT-PCR for Massachusetts, D274, and 4/91 types (1) using Platinum Taq DNA Polymerase (Applied Biosystems) per the manufacturer's instructions.

RT-PCR for the S1 region. To better characterize the coronavirus strains detected in quail, all 3′-UTR–positive RNA samples were submitted to an RT-PCR targeted to the S1-coding region in the S-gene by using primers and cycling conditions described by Worthington *et al.* (30) and Platinum Taq DNA Polymerase per the manufacturer's instructions. The expected 390-bp amplicons were then submitted to DNA sequencing and phylogenetic analysis.

Cloning of PCR products. To increase the concentration of DNA for 3′-UTR amplicons of quail samples, PCR products were cloned into plasmids before DNA sequencing. The 179-bp bands were purified using the GFX™ kit (GE Healthcare, Fairfield, CT) and individually inserted into pTZ57R/T plasmids by using InsTaclone™ (MBI Fermentas, Hanover, MD) according to manufacturer's instructions and used to transform competent JM 109 *Escherichia coli.* Two JM 109 clones for each amplicon were submitted to plasmid extraction by using a NucleoSpin Plasmid™ kit (Macherey Nagel, Düren, Germany) as recommended by the manufacturer. After the extraction, the presence of the inserted amplicons in each clone was checked with PCR by using primers M13/pUC forward and M13/pUC reverse targeted to the insertion flanking regions of the plasmid, according to the manufacturer's instructions.

DNA sequencing and phylogenetic analyses. Amplicons, including those inserted in plasmids, were purified from agarose gels by using the GFX™ kit (GE Healthcare) and submitted to bidirectional DNA sequencing with Big Dye™ 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI-3500 automatic sequencer (Applied Biosystems).

Sequences with Phil's Read Editor scores >20 (16) were assembled and analyzed with Cap-Contig application included in Bioedit 7.0.9.0 software (18), with homologous sequences retrieved from GenBank (for accession numbers, see Figs. 1, 2).

A nucleotide tree (neighbor-joining, maximum composite likelihood model, 1000 bootstrap replicates) and amino acids tree (neighbor-joining, Poisson correction, 1000 bootstrap replicates) of each genome region (3′-UTR and *RdRp*) were built using MEGA 5.0 software (25).

Statistical analysis. The association between the species of bird (quail and laying hens) and the presence of the virus was determined by

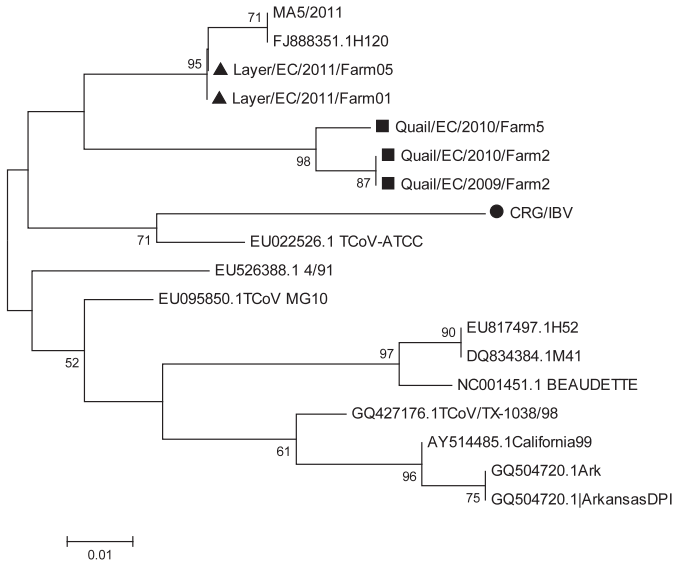

Fig. 2. Phylogenetic distance tree with the neighbor-joining algorithm for the RdRp nucleotide (nt 15,647–15,826) showing classic serotypes/genotypes (with GenBank accession numbers) and samples included in this study (squares for quail, triangles for laying hens, and a circle for chicken CGR strain isolated from a poultry flock). The numbers above each node represent the bootstrap values for 1000 replicates (only values >50% are shown). The bar represents the number of nt substitutions per site.

applying the Fisher exact test with MINITAB® 15.1.0.0. (Minitab Inc., State College, PA).

RESULTS AND DISCUSSION

In this investigation, an *Avian coronavirus* was detected in five of six farms of quail (14 flocks) and laying hens (four flocks), including farms with detection in both host species simultaneously, from birds with and without clinical signs compatible with IB. Viral RNA was detected in pools of female reproductive tracts, lungs, kidneys, tracheas, and enteric contents of quail and in lungs, kidneys, and tracheas of layers.

In 2010, regarding the six quails' and four layers' pools positive for *Avian coronavirus*, a higher frequency was found in the reproductive tracts (9%) and tracheas (18.2%) from quail compared with those from layers (0% in the reproductive tract and 9% in tracheas). One of the quail-positive pools of tracheas belongs to a flock of birds that were 2 mo old presenting gasping and watery eyes, similar symptoms to those observed in chickens at few days or weeks old (5,10).

Avian coronavirus also has been found in samples of quail reproductive organs that had production drop and egg alterations as well as signs of false layers. In the reproductive tracts of laying hens, the most common manifestation of IBV infection is evidenced in the production and quality of eggs because of viral replication in the oviducts that can cause permanent damage to the development in young females. This damage can lead to partial or complete atrophy of the ovaries, and the affected birds may ovulate normally without egg production, thus becoming false layers (5). Thus, it is likely that in quail, the pathogenesis of infection by *Avian coronavirus* in the reproductive tract is similar to that already described in chickens, leading to the signs observed in the studied farms.

Kidney pools of layers had a higher incidence (27.2%) of *Avian coronavirus* compared with quail (18.2%), whereas lung pools for both species had the same incidence (9%). The finding of *Avian coronavirus* in pools of kidneys of quail and chickens without apparent gross pathologic changes may be related to this virus replicating not only in respiratory tissues but also in kidneys, where there may be chronic infection (11,14). Fisher test showed no statistically significant difference between layers and quail regarding avian coronavirus incidence ($P = 1.0$).

Concerning the enteric contents samples of quail in which *Avian coronavirus* was found, this incidence could suggest that the fecal-oral/respiratory route is relevant to quail populations as suggested for chickens (28) and geese (19) or even that caecal tonsils could be a possible site of persistent infection (4,12,24,28).

aMPV was not detected in any quail samples; however, in one pool of reproductive tracts and lungs of laying hens, it was possible to detect subtype B metapneumovirus. NDV was not detected in any samples.

All strains of IBV detected in samples from layers and quail are different from genotypes Massachusetts, D274, and 4/91 as determined by the typing RT-PCR. IBV types other than the archetypical types are widespread in breeders, broilers, and layers in Brazil, possibly because of the continuous use of attenuated vaccines solely based on the Massachusetts serotype (29).

Sequences of 97 nucleotides (3′-UTR) and 116 nucleotides (*RdRp*) from quail samples of enteric contents (Farms 2, 4, and 5) and kidneys (Farm 4) and samples of lungs, tracheas, kidneys, and enteric contents belonging to laying flocks from different regions of Brazil were used to generate sequences for phylogenetic analyses (Table 1).

The 3′-UTR tree (Fig. 1) showed that viruses of both quail and chickens segregated with other avian coronaviruses, including turkey coronavirus, and viruses from pheasants (*Phasianus colchicus*) and peacocks, indicating that these viruses are IBV-like gammacoronaviruses. There is a trend to a phylogeographic pattern, as two lineages of *Avian coronavirus* in quail were found in two different farms located in the region of Bastos, SP. In general, these results agree with the *RdRp* tree topology (Fig. 2), but with lower resolution. Because sequences were <200 nucleotides, they were not submitted to GenBank, but they are available upon request.

Mean nucleotide identity between the 3′-UTR for the group of quail was 98.7%, 97.5% between quail and laying hens, and 96.4% with the Massachusetts genotype (H120). Sequences from layer samples were 100% identical amongst each other and 98.9% identical to H120.

Nucleotide identity of *RdRp* between quail groups was 98.8%, 92.13% between quail and layers, and 93.1% with the Massachusetts genotype (H120 and Ma5). Sequences from layer samples were 90.5% identical amongst each other and 95.3% identical to genotype Massachusetts (H120 and Ma5).

Nonetheless, any accurate distinction between vaccine or archetypical IBV strains and field strains must be based on S-gene analyses because the 3′-UTR and the *RdRp* gene do not show reliable molecular markers to this end. Because no sequences were obtained for this region for the quail strains, due either to a low virus titer in the samples or to polymorphisms in the primers' annealing sites, the exact type of avian coronavirus in these birds remains to be determined after the isolation of these viruses in chicken embryos followed by DNA sequencing of the complete S- and M-genes.

Whether quail are amplifiers of wild IBV types that can cause disease in chickens, or the avian coronavirus strains reported herein are restricted to quail and can indeed induce disease in these birds, is a matter for future research, based on the isolation of these viruses and experimental inoculation in quail and chickens with homologous and heterologous isolates.

Table 1. Strains include in the analysis of the 3′-UTR and *RdRp* gene according to geographic region of origin (ES, Espítito Santo; MG, Minas Gerais; RS, Rio Grande do Sul; SC, Santa Catarina; SP, São Paulo), bird species (quail and layer), clinical signs at the sampling time, and type of sample.

Strain	State	Host	Signs	Sample
Quail/2009/EC/Farm2[A]	ES	Quail	Rep[B]	Enteric contents
Quail/2O1O/EC/Farm2[A]	ES	Quail	Rep	Enteric contents
Quail/2010/EC/Farm5[A]	SP	Quail	Res,[C] Rep	Enteric contents
Quail/2010/EC/Farm5.1[A]	SP	Quail	Res, Rep	Enteric contents
Quail/2010/EC/Farm5.2[A]	SP	Quail	Res, Rep	Enteric contents
Quail/2010/K/Farm3[A]	SP	Quail	Rep	Kidneys
Quail/2010/EC/Farm3[A]	SP	Quail	Rep	Enteric contents
Layer/2011/EC/Farm01	PR	Layers	Res	Enteric contents
Layer/2011/EC/Farm05	RS	Layers	Res	Enteric contents
Layer/2011/K/Farm04	MG	Layers	Res, Rep	Kidneys
Layer/2011/L/Farm09	SC	Layers	NA[D]	Lungs
Layer/2011/TR/Farm16	SP	Layers	Rep	Tracheas and lungs

[A]Samples from farms collected in 2009 and 2010.
[B]Reproductive.
[C]Respiratory.
[D]NA = not available.

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ACKNOWLEDGMENTS

We are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support (grant 132912/2010-4) and to the field veterinarians and farmers who submitted the samples. We also thank Prof. Dr. Ricardo Augusto Dias for advice with the statistical analyses.