CAROLINA TORRES ALEJO

Study of the role of quail as reservoirs for avian infectious bronchitis virus

São Paulo
2015

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Tese apresentada ao Programa de Pós-Graduação em Epidemiologia Experimental Aplicada às Zoonoses da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo - Brasil e Faculdade de Agricultura e Medicina Veterinária da Universidade de Pádua - Itália para obtenção da Dupla Titulação de Doutor em Ciências

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Prof. Dr. Paulo Eduardo Brandão

Prof. Dr. Mattia Cecchinato

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Comissão de Ética no uso de animais

CERTIFICADO

Certificamos que o Projeto intitulado "Estudo do papel das codornas como reservatório para a bronquite infecciosa das galinhas", protocolado sob o nº 2697/2012, utilizando 80 (oitenta) aves, sob a responsabilidade do(a) Prof. Dr. Paulo Eduardo Brandão, está de acordo com os princípios éticos de experimentação animal da "Comissão de Ética no uso de animais" da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo e foi aprovado em reunião de 17/10/2012.

We certify that the Research "Study of the role of quail as reservoir for avian infectious bronchitis", protocol number 2697/2012, utilizing 80 (eigthy) birds, under the responsibility Prof. Dr. Paulo Eduardo Brandão, agree with Ethical Principles in Animal Research adopted by "Ethic Committee in the use of animals" of the School of Veterinary Medicine and Animal Science of University of São Paulo and was approved in the meeting of day 10/17/2012.

São Paulo, 18 de outubro de 2012.

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A mi familia por todo el amor y apoyo incondicional.
Sin ustedes nada sería posible.

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RESUMO

TORRES, C. A. **Estudo do papel das codornas como reservatório para o vírus da bronquite Infecciosa das galinhas.** [Study of the role of quail as reservoirs for avian infectious bronchitis virus]. 2015. 53 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2015. Tese (Doutorado em Ciências Veterinárias) - Faculdade de Agricultura e Medicina Veterinária da Universidade de Pádua, Pádua, 2015.

Este estudo teve como objetivo pesquisar a ocorrência e diversidade molecular de coronavírus em codornas e galinhas criadas nas mesmas propriedades e em codornas criadas em propriedades isoladas, para determinar o papel das codornas como reservatório para o vírus da bronquite infecciosa das galinhas (IBV). Para isso, duas pesquisas foram realizadas, uma em 2013, no estado de São Paulo, Sudeste do Brasil, onde algumas granjas iniciaram a vacinação em codornas contra o IBV com o sorotipo Massachusetts, após um estudo realizado em 2009-2010; e a outra, em 2015, em duas regiões do Norte da Itália. No estudo brasileiro, foram coletados *pools* de aparelho reprodutor, pulmões, rins, traqueia e conteúdo entérico de codornas (*Coturnix coturnix japonica*) e galinhas com histórico de manifestações clínicas compatíveis com a Bronquite Infecciosa das galinhas (BIG). Por outro lado, no estudo italiano, as amostras foram coletadas em forma de *pools* de *swabs* traqueais e cloacais e intestino/conteúdo entérico de codornas (*Coturnix coturnix*) com sinais entéricos. Estas amostras foram testadas para os coronavírus aviário (*Gammacoronavirus)* mediante uma *semi-nested* RT-PCR dirigida a região não-traduzida 3' (3´UTR). As amostras positivas foram submetidas a RT-PCR do gene codificador da proteína RNA-polimerase RNA-dependente (*RdRp*) e duas RT-PCRs, incluindo uma multiplex dirigidas a proteína de espícula (S) do vírus da BIG, para genotipagem. Além disso, a detecção de metapneumovírus aviário (aMPV) e o vírus da doença de Newcastle (NDV) também foi realizada por meio de RT-PCRs. Coronavírus aviários foram encontrados em todos os tipos de amostras estudadas em codornas e galinhas de todos os tipos de criações, aMPV subtipo B foi encontrado em galinhas (Brasil) e o NDV não foi encontrado em nenhuma amostras. Com base nas sequências de DNA para o gene codificador da proteína *RdRp,* as amostras brasileiras e italianas foram agrupadas no gênero *Gamma*-

ou *Deltacoronavirus*, enquanto que, em uma amostra brasileira, foi detectada co-infecção pelos dois gêneros. A filogenia com base nas sequências parciais da subunidade S1da proteína de espícula, evidenciou que os *Gammacoronavirus* detectados nas codornas brasileiras e italianas pertencem ao genótipo Brasil e 793/B, respectivamente. Estes resultados sugerem que as codornas são suscetíveis aos coronavírus do gênero *Gamma* e *Delta* e os coronavírus aviários das codornas compartilham genes de espícula idênticos aos do IBV. Desta forma, sugere-se que as codornas podem servir como reservatórios para coronavírus aviários e que a vacinação com o sorotipo Massachusetts não foi eficiente no controle de IBV nas codornas brasileiras.

Palavras-chave: Coronavírus aviário. *Deltacoronavirus.* Codornas. Galinhas. Reservatório.

ABSTRACT

TORRES, C. A. **Study of the role of quail as reservoir for avian infectious bronchitis virus.** [Estudo do papel das codornas como reservatório para o vírus da bronquite infecciosa das galinhas]. 2015. 53 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2015. Tese (Doutorado em Ciências Veterinárias) - Faculdade de Agricultura e Medicina Veterinária da Universidade de Pádua, Pádua, 2015.

This study aimed to investigate the occurrence and molecular diversity of coronavirus in quail and laying hens raised on the same farms and quail only farms, to determine the role of quail as reservoir for avian infectious bronchitis virus (IBV). To this end, two investigations were carried out, one in the São Paulo state, Southeastern Brazil, in 2013, when some farmers started quail vaccination with Massachusetts IBV serotype after surveillance carried out in 2009-2010 and the other in two regions of Northern Italy, in 2015. In the Brazilian study, samples were collected as pools of tracheas, lungs, reproductive tract, kidneys and enteric contents from quail (*Coturnix coturnix Japonica*) and laying hens showing IB-like symptoms, while, in the Italian study, samples were collected as pools of tracheal and cloacal swabs and intestine/enteric content from European quail (*Coturnix coturnix*), showing enteric disorders. All samples were tested by a nested RT-PCR targeted to the 3'UTR of the *Gammacoronavirus* genus. Positive samples were submitted to RT-PCR to the RNA-dependent RNA-polymerase gene (*RdRp*) and two different RT-PCRs to the spike gene, including a typing-multiplex one. Two other RT-PCRs were used to detect the avian metapneumovirus (aMPV) and Newcastle disease virus (NDV). *Avian coronavirus* was found in all types of samples analyzed in quail and chickens from both type of creations, aMPV subtype B was found in chickens (Brasil) and the NDV was not observed in any samples. Based on the DNA sequences for the *RdRp* gene, Brazilian and Italian quail strains clustered within either *Gammacoronavirus* or *Deltacoronavirus* genus, while, for one Brazilian sample, it was detected co-infection with the two genuses. Phylogeny based on partial S1 subunit sequences showed that the gammacoronaviruses detected in the Brazilian and Italian quail belong to the Brazil type and 793/B, respectively. These results suggest that quail are susceptible to G*amma* and *Deltacoronavirus* and that quail avian coronavirus share

spike genes identical to chicken infectious bronchitis virus (IBV); thus, quail might act as reservoirs for avian coronaviruses. Also, Massachusetts vaccination was not efficient to control IBV in Brazilian quail.

Keywords: *Avian coronavirus*. *Deltacoronavirus*. Quail. Chickens. Reservoir.

ABSTRACT

TORRES, C. A. **Studio del ruolo della quaglia come reservoir per il virus della bronchite infettiva.** [Estudo do papel das codornas como reservatório para o vírus da bronquite infecciosa das galinhas]. 2015. 53 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2015. Tese (Doutorado em Ciências Veterinárias) - Faculdade de Agricultura e Medicina Veterinária da Universidade de Pádua, Pádua, 2015.

Lo scopo di questo lavoro è valutare il ruolo della quaglia come reservoir per il virus della bronchite infettiva (IBV), verificando la presenza del coronavirus in quaglie allevate da sole e in quelle allevate in allevamenti che hanno anche capannoni di galline ovaiole, e valutando eventuali differenze nelle sequenze genetiche isolate nei due gruppi. A questo scopo sono stati condotti due studi, uno nello stato di San Paulo, sudest del Brasile, nel 2013, quando, dopo un monitoraggio svolto nel 2009-2010, alcuni allevatori avevano cominciato la vaccinazione delle quaglie con IB sierotipo Massachusetts, e l'altro condotto in altre due regioni del Nord Italia. Nello studio condotto in Brasile, I campioni sono stati raccolti in pool di trachee, polmoni, tratto riproduttivo, reni e contenuto intestinale da quaglie (*Coturnix coturnix Japonica*) e galline ovaiole che mostravano sintomi compatibili con IB. Invece nello studio condotto in Italia sono stati raccolti pool di tamponi tracheali e cloacali e di contenuto intestinale di quaglie europee (*Coturnix coturnix*) che presentavano sintomatologia enterica. Tutti i campioni sono stati analizzati con una nested RT-PCR disegnata sulla sequenza 3'UTR del gene *Gammacoronavirus*. I campioni positivi sono stati sottoposti ad una RT-PCR per il gene RNA-dependent RNA-polymerase (*RdRp*) e a due diverse RT-PCR per il gene S, inclusa una multiplex per genotipizzare. Altre due RT-PCR sono state usate per la ricerca di Metapneumovirus Aviare (aMPV) e virus della malattia di Newcastle (NDV). Il coronavirus aviare è stato trovato in tutti i campioni analizzati di galline ovaiole e quaglie in entrambi i tipi di allevamento, aMPV sottotipo B è stato riscontrato nelle galline ovaiole (Brasile), mentre NDV non è mai stato trovato. Sulla base delle sequenze di DNA del gene *RdRp*, i ceppi isolati dalle quaglie in Brasile ed Italia clusterizzano o con il genere *Gammacoronavirus* o col genere *Deltacoronavirus*, mentre un solo campione brasiliano presenta sequenze di entrambi i generi (coinfezione). L'analisi filogenetica

basata su sequenze parziali della subunità S1mostra che i gammacoronavirus trovati nelle quagli brasiliane e italiane appartengono rispettivamente ai ceppi brasiliano e 793/B. Questi risultati suggeriscono che le quaglie sono suscettibili sia ai *Gamma* che ai *Deltacoronavirus* e che il coronavirus aviare della quaglia condivide geni S con il virus della bronchite infettiva del pollo (IBV); dunque la quaglia può fungere da reservoir per i coronavirus aviari. Inoltre la vaccinazione con ceppo Massachusetts si è rivelata inefficace per il controllo di IBV nella quaglia.

Parole chiave: Coronavirus aviare. *Deltacoronavivirus*. Quaglia. Gallina ovaiola. Reservoir

SUMÁRIO

1 INTRODUCTION

Coronaviruses (CoVs), which name derives from *corona* due to the crown-like appearance observed for these viruses in the electron microscope, belong to the *Nidovirales* order and *Coronaviridae* family, which includes two subfamilies, *Torovirinae* and *Coronavirinae*. The *Coronavirinae* is further subdivided into the genera *Alpha*-, *Beta*- *Gamma*- and *Deltacoronavirus* (ICTV, 2014; FEHR; PERLMAN, 2015) which are divided by phylogenetic clustering (GORBALENYA et al., 2006; FEHR; PERLMAN, 2015).

The genera *Alpha*- and *Betacoronavirus* are found exclusively in mammalian species including bats to humans (WENTWORTH; HOLMES, 2007), while *Gammacoronavirus* contains the species Beluga Whale coronavirus (BWCoV) and *Avian coronavirus*, which is now a single viral species containing the previously named Avian infectious bronchitis virus (IBV) in chickens and Turkey coronavirus (TCoV); throughout this thesis, *Avian coronavirus* will be used to refer to the *Gammacoronavirus* species in general, while *IBV* might be used sometimes regarding *Avian coronavirus* from chickens.

Gammacoronaviruses were also described in pheasants (PhCoV), ducks (DCoV), pigeons (PCOV), peafowl, teals, quail and in several species of wild birds (CAVANAGH, 2001; CAVANAGH, 2005; LIU et al., 2005; JONASSEN et al., 2005; FELIPPE et al., 2010; CIRCELLA et al., 2007; CARDOSO et al., 2011; TORRES et al., 2013). Even more, detection of *Gamma* and *Deltacoronavirus* in wild birds were also reported by Chu et al. (2011), Durães-Carvalho et al. (2015) and Jordan et al. (2015).

In the last decade, several researches on coronavirus have been conducted allowing the discovery of new viral species and lineages and the phylogenetic analysis of known genomes.

Bats are the source of an wide variety of alpha- and betacoronaviruses, enhancing interspecies transmission (WOO et al., 2012), as the case of the emergence of Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) and Middle East Respiratory Syndrome coronavirus (MERS-CoV), in which two strains of animal coronaviruses crossed the species barrier to infect and cause severe respiratory infections in humans (GRETEBECK; SUBBARAO, 2015). On the other hand, birds are the gene source of Gamma- and deltacoronaviruses that most likely infected birds and were later introduced into some mammals (WOO et al., 2012).

Coronaviruses contain a up to 32kb-long, non-segmented, positive-sense RNA genome, which is 3' polyadenylated and has a 5' cap structure, then acting as an mRNA for translation of the replicase polyproteins (HAGEMEIJER; ROTTIER; DE HAAN, 2012; FEHR; PERLMAN, 2015).

The 5' and 3' ends of the genome contain untranslated regions (UTRs) that are important for replication and transcription (MASTERS, 2006; HAGEMEIJER; ROTTIER; DE HAAN, 2012). Two-thirds of the genome consists of two large open reading frames (ORFs), ORF1a and ORF1b that encode replicase-transcriptase proteins, which are synthesized as two large polyproteins (pp1a and pp1ab) by a programmed ribosomal frameshifting (NAMY et al., 2006). These polyproteins undergo autoproteolytic cleavage into 15 or 16 non-structural proteins (nsps) (ZIEBUHR; SNIJDER; GORBALENYA, 2000) that build up the viral RNA replication complex (GORBALENYA et al., 2006). Furthermore, transcriptional regulatory sequences (TRSs) are located at the beginning of each structural or accessory gene and are necessary for their transcription. All known coronaviruses have the same sequence of ORFs arranged as 5´UTR-replicase-S (Spike)- E (Envelope) –M (Membrane)- N (Nucleocapsid)- 3´UTR-poly (A) within the structural genes at the 3′ end of the genome (FEHR; PERLMAN, 2015). Some betacoronaviruses contain an additional membrane protein, a hemagglutinin-esterase (HE) (REGL et al., 1999).

The S protein is a class I transmembrane protein which forms homotrimers in the viral envelope (WEISS; NAVAS-MARTIN, 2005) and has two functional domains on the

large ectodomain. One (S1), at the amino-terminal end, mediates attachment and binding to a receptor, cell tropism, the host species specificity, pathogenicity, induction of neutralizing antibodies and cellular immune response and emergency types variants in the case of *Avian Coronavirus* (CASAIS et al., 2003; MCKINLEY; HILT; JACKWOOD, 2008; MONTASSIER et al., 2010; JACKWOOD et al., 2012), whereas the second one (S2) is responsible for the fusion of the viral and the host cell membrane (CAVANAGH, 2005).

The M protein is the most abundant structural protein in the virion and is essential for the overall structure of the viral particle by determining the position of its components. Furthermore, the combined expression of M and E protein, involved in the assembly, results in the formation of virus like particles (CAVANAGH, 2007).

The N protein makes the nucleocapdis and facilitates the RNA synthesis, being indispensable for viral assembly. Also, play an important role in cell-mediated immunity and probably facilitates the transport of the viral genome from the synthesis site to the site of formation of the viral particles (VERHEIJE et al., 2010).

The CoV nsps form together with the nucleocapsid (N) protein, and presumably several host proteins, the membrane-associated replication-transcription complexes (HAGEMEIJER; ROTTIER; DE HAAN, 2012). The most conserved and important nonstructural protein involved in genome replication is RNA polymerase RNA-dependent (RdRp) (nsp12) (HAGEMEIJER; ROTTIER; DE HAAN, 2012), product of the open reading frame (ORF) 1ab. It is responsible for transcription and for viral genome replication, being part of the replicase complex, which is also responsible for the discontinuous transcription process that leads to generation of a set of co-terminal subgenomic mRNAs (STEPHENSEN; CASEBOLT; GANGOPADHYAY, 1999; SAWICKI; SAWICKI; SIDDELL, 2007).

The first description of a virus similar to coronavirus in quail was by Pascucci et al. (1983) from isolated samples of birds with a respiratory syndrome. In 1985, the same group of researchers using virus neutralization test and Immunoelectron Microscopy

showed that one of the lineages was not related to IBV coronavirus and other mammals CoVs (PASCUCCI et al. 1985).

In Brazil, Di fabio et al. (2000) isolated an IBV strain from healthy and non-vaccinated quail, showing a low antigenic identity with IBV serotypes Massachusetts, Connecticut, and D207. Later, in 2005, an enteric syndrome in quail farms located in southern Italy was described by Circella et al. (2007); based on the RdRp gene, showed a 16-18% identity with IBV, suggesting that specifically QCoV was not an IBV variant and it was proposed that the detected coronavirus belonged to a new avian CoV specie denominated Quail coronavirus (QCoV). In Italy, IBV strains as the Italy/624I/94, 793/B, B1648, Massachusetts (CAPUA et al., 1999) and Italy-02 (Italy/Italy-02/ 497/02) type were reported (WORTHINGTON; CURRIE; JONES, 2008). Moreover, the QX– like IBV types (BEATO et al., 2005; WORTHINGTON; CURRIE; JONES, 2008) and a Q1-like IBV were detected in this country (FRANZO et al., 2015), showing the circulation of several IBV lineages.

In 2012, the study conducted by Torres et al. (2013) demonstrated that avian coronaviruses in symptomatic/asymptomatic quail are closely related to IBV based on the RdRp and the 3´UTR partial DNA sequences. After the IBV surveillance carried out in 2009-10, some quail farmers began to vaccinate quail against IBV with live attenuated or inactivated vaccines based on the Massachusetts (Mass) serotype; nevertheless IB outbreaks continued to occur on vaccinated flocks, caused by Brazilian genotypes (BR) divergent from the vaccine strain, as previously report in chickens (ABREU et al., 2006; VILLARREAL et al., 2007; MONTASSIER, 2010; FELIPPE et al., 2010; VILLARREAL et al., 2010; CHACON et al., 2011; BALESTRIN et al., 2014).

Furthermore, Caserta et al. (2014) isolated an A*vian Coronavirus* of the Massachusetts genotype, probably form a vaccine origin, from a quail farm with high mortality caused by a nutritional disorder and decrease in egg production.
In the Brazilian poultry flocks, besides the predominance of the BR genotypes (DE WIT et al., 2015), IBV strain similar to genotype 4/91 was also described (VILLARREAL et al., 2010).

Data gathered thus far does indicate that quail are susceptible to *Avian coronaviruses*, but the gene pool of these viruses in this avian host and its occurrence under different epidemiological scenarios is still poorly known.

Based on the evidence demonstrated in previous studies, the hypothesis of this investigation was that quail and chickens share avian coronaviruses closely related, keeping an epidemiological cycle that increases the viral diversity and challenge remains constant for these host populations.

2 OBJETIVES

The objectives of this Thesis were:

1. To evaluate the occurrence of coronaviruses in Brazilian and Italian quail farms with or without clinical signs in quail housed with chickens and quail only farms.
2. Assess the molecular diversity of coronaviruses found in quail after the introduction of vaccination programs in some quail farms in Brazil and farms where the quail are not vaccinated.

3 *GAMMA AND DELTACORONAVIRUS* CO-INFECTION IN QUAIL WITH RESPIRATORY AND REPRODUCTIVE SIGNS

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SUMARY

This paper expands a previous report on coronavirus in quail. To this end, samples were collected from São Paulo state, Southeastern Brazil, in 2013, when some farmers started quail vaccination with Massachusetts IBV serotype after surveillance carried out in 2009-2010. Pools of tracheas, lungs, reproductive tract, kidneys and enteric contents from quail and laying hens kept in the same farms and quail-only farms, showing IB-like symptoms, were sampled in this study. Samples were tested by a nested RT-PCR targeted to the 3'UTR of the *Gammacoronavirus* genus. Based on the DNA sequences for the RdRp gene, quail strains clustered within either *Gammacoronavirus* or *Deltacoronavirus* genus, while, for one quail sample, sequences from both genera were found. Phylogeny based on partial S1 subunit sequences showed that the gammacoronaviruses detected in quail and layers belong to the Brazilian type. These results not only suggest that quail are susceptible to G*amma* and *Deltacoronavirus*, but also that Massachusetts vaccination was not efficient to control IBV in quail, as well as in chickens.

Keywords: Coronaviruses. Quail. Laying hens. IBV. *Gammacoronavirus*. *Deltacoronavirus*.

Abbreviations: aMPV = Avian metapneumovirus, DEPC = diethyl pyrocarbonate, IBV = Avian infectious bronchitis virus, IB = Infectious bronchitis, NDV = Newcastle disease virus, PHRED = Phil's Read Editor, QCoV = Quail coronavirus, *RdRp* = RNA- dependent RNA polymerase, RT-PCR = reverse transcription-polymerase chain reaction, S-gene = spike gene, UTR = untranslated region, v/v = volume/volume

INTRODUCTION

In Brazil, after an *Avian coronavirus* (Avian infectious bronchitis virus, IBV) surveillance carried out in 2009-10 (Torres et al., 2013), some quail farmers began to vaccinate quail against IBV with live attenuated or inactivated vaccines based on the Massachusetts (Mass) serotype. Nevertheless, infectious bronchitis (IB) outbreaks continued to occur on vaccinated flocks, caused by Brazilian field strains of a serotype divergent from the vaccine strain, as previously report in chickens (Abreu et al., 2006; Villarreal et al., 2007; Montassier, 2008; Montassier, 2010; Felippe et al., 2010; Villarreal et al., 2010; Chacon et al., 2011; Balestrin et al., 2014). *Avian coronavirus* (IBV in chickens), the causative agent of IB, is a *Gammacoronavirus* that can replicate on every epithelial surface, causing economically important poultry disease affecting renal, reproductive and respiratory systems and causing enteritis (Cavanagh, 2003; Cavanagh, 2005; Villarreal et al., 2007; Jones, 2010). 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 (Liu et al., 2005).

Besides IBV, coronaviruses from the *Gamma* (*γ-CoV*) and *Deltacoronavirus* (*δ-CoV*) genera are also detected in asymptomatic birds (Liu et al., 2005; Jonassen et al., 2005; Felippe et al., 2010; Cardoso et al., 2011; Woo et al., 2009; Chu et al., 2011; Durães-Carvalho et al., 2015; Jordan et al., 2015).

This investigation aimed to study the molecular diversity of coronaviruses in quail (C*oturnix coturnix japonica*) housed in proximity to laying hens and quail-only farms, in order to understand the viral diversity that remains a constant challenge for these host populations, despite vaccination.

MATERIALS AND METHODS

Source of viruses. In 2013, samples were collected from four farms, being two quail-layers farms previously sampled during 2009-10 and identified as Quail-layers Farm1 and Farm2, and two quail-only farms (Quail Farm3 and Farm4) in the state of São Paulo, municipalities of Bastos and Iacri, in southeastern Brazil. The flocks showed disorders of respiratory and reproductive tracts including nasal discharge, gasping,

watery eyes, conjunctivitis, egg production drop, eggs with thin-walled and misshapen shells with loss of pigmentation.

Chickens had been vaccinated against IBV using attenuated plus inactivated Massachusetts vaccines, against Avian Metapneumovirus (aMPV) with subtype A or B and against Newcastle Disease Virus (NDV) with a lentogenic vaccine. In two farms, quail were vaccinated against IBV, one of them (Quail Farm4) using the same vaccination schedule as in chickens and the other (Quail-layers Farm2) just an attenuated Massachusetts vaccine. All quail were vaccinated with the lentogenic Newcastle disease virus (NDV) but not against *Avian metapneumovirus* (aMPV).

Samples were collected as pools of female reproductive tract, lungs, kidneys, tracheas and complete enteric contents from 18 flocks (12 for quail and 6 for chickens) from five birds (quail or chickens) per house with and without clinical signs in a total of 90 samples.

Controls. The IBV Massachusetts strain vaccine (NOBILIS™ IB Ma5; MSD Animal Health, Boxmeer, The Netherlands), aMPV strain vaccine RTV 8544 (NOBILIS™ RTV 8544; MSD Animal Health, Boxmeer, The Netherlands) and NDV lentogenic strain vaccine (ND LA SOTAL NOBILIS™; MSD Animal Health, Boxmeer, The Netherlands) were used as positive controls for reverse transcription-polymerase chain reaction (RT-PCR). As negative controls, ultra pure water treated with 0.1% diethyl pyrocarbonate (DEPC) was included.

RNA extraction. Pools of organs and enteric content were prepared as 50% (v/v) suspensions in diethyl pyrocarbonate (DEPC)-treated water and submitted to three freeze-thaw cycles in liquid nitrogen and 56°C and clarified at 5,000g x for 15 min at 4°C. Total RNA was extracted from the supernatants with Trizol Reagent™ (Life Technologies Brazil) according to the manufacturer's instructions.

Synthesis of complementary DNA (cDNA). 3.5 µl of each RNA were denatured at 94 ºC/5 min, and reverse-transcribed with Random Primers^{TM} and M-MLV Reverse Transcriptase^{TM} (Life Technologies Brazil) as per manufacturers. For partial amplification of the pan-coronavirus RdRp gene and S gene of IBV, reverse transcription

steps were carried out with specific primers and ThermoScript™ RT (Life Technologies Brazil).

***Avian coronavirus*, aMPV and NDV screening**. Each sample was screened for IBV, aMPV and NDV as described by Cavanagh et al. (2002), Cavanagh et al. (1999) and Tiwari et al. (2004), targeting the 3´-untranslated region (UTR), G gene and F gene, respectively, using GoTaq™ Green Master Mix (Promega Brazil) as per manufacturer's instructions, with amplicons of 179, 268 or 361 (aMPV types A or B) and 255 bp, respectively.

RNA-dependent RNA polymerase (*RdRp*) and Spike gene sequence analysis. 3'UTR- *Avian coronavirus* positive samples were then tested by a pan-coronavirus RT-PCR targeting the RNA- dependent RNA polymerase (*RdRp*) gene as described by Chu et al. (2011) (440bp amplicon), by a typing S-gene targeted multiplex RT-PCR for Massachusetts, D274 and 4/91 types (Capua et al. 1999) and a pan-IBV S1 gene (*Avian coronavirus*) RT-PCR described by Torres et al. (2014) (450bp amplicon).

Amplicons were purified from agarose gels with the GFX PCR DNA and Gel Band purification Kit™ (GE Healthcare, UK), visually quantified by Low DNA Mass Ladder™ (Invitrogen, USA) according to the manufacturer's instructions and submitted to bidirectional DNA sequencing with Big DyeTM 3.1 (Cycle Sequencing Kit, Applied Biosystems™, USA) and an ABI-3500 Genetic Analyzer (Applied Biosystems™, USA).

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 Cap-Contig application included in Bioedit 7.0.9.0 software (Tom Hall © 1997-2007) and aligned with homologous sequences retrieved from GenBank (accession numbers in Figure 1 and 2). Nucleotide (Neighbor-joining, Maximum Composite Likelihood model, 1,000 bootstrap replications) and amino acids trees (Neighbor-Joining, Poisson correction, 1,000 bootstrap replications) for S gene and *RdRp* were built using MEGA 5.2.1 (Tamura et al., 2011).

Cloning of PCR products. In order to solve dubious chromatograms peaks for RdRp amplicons in two quail samples from Quail-layers Farm2 and Quail Farm3, PCR products were cloned into plasmid prior to DNA sequencing with primers RdRp2Foward and RdRp2Reverse as described above. Briefly, 440-bp amplicons were purified using the GFX^{TM} kit (GE Healthcare, UK) and individually inserted into pGEM^{TM}-5Zf (+) plasmids (pGEM^{TM}-T Vector System II™, Promega, USA), according to manufacturer's instructions and used to transform competent JM 109 *Escherichia coli*. 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.

Virus isolation. Four quail coronavirus-positive pools (kidneys, enteric content, reproductive tract and lungs + trachea), one from each farm, and negative for aMPV and NDV, were used for virus isolation as described by Gelb & Jackwood, (2008). On day 3 post-inoculation, three eggs per sample were removed from the incubator and placed at 4°C for 18 hr. Chorioallantoic fluids of inoculated eggs were harvested and stored at -80ºC until RNA extraction, using the PureLink^{TM} RNA Mini Kit (Ambion, Brazil). The remaining eggs were incubated up to 7 days and observed for typical IBV lesions. Four blind serial passages were performed at the same way, to complete five blind passages per sample. Virus isolation was monitored with the screening PCRs for all viruses described above using the allantoic fluids as samples. PBS was included as negative control.

Statistical analysis. The association between the species of bird (quail and laying hens) and the presence of the virus was determined by applying the Fisher exact test with Minitab 15.1.0.0. (© 2006 Minitab Inc.). The frequency of occurrence of the coronavirus in quail and laying hens was determined by the ratio between the number of positive samples by the hemi-nested RT-PCR for the 3'UTR for each class of samples and all samples tested in each class.

RESULTS AND DISCUSSION

In this investigation, *Gammacoronavirus* was detected in all studying farms. As previously reported by Torres et al. (2013), *Avian coronavirus* closely related to infectious bronchitis virus has been identified in the studied area, since at this time 88% (16/18) of the flocks were considered positive, including detection in quail (46.6%; 28/60) and chickens (20%; 6/30) pools simultaneously, from birds with and without clinical signs compatible with infectious bronchitis.

Taking into account the division by pools, 41.6% (5/12) of kidneys and reproductive tract, 8.3% (1/12) of the lungs, 58.3% (7/12) of tracheas and 83 3% (10/12) of enteric contents of quail were positive for *Gammacoronavirus*. As for the chickens, viral RNA was identified in pools of enteric contents (83.3%; 5/6) and kidney (16.6%; 1/6). aMPV was not detected in any quail samples; however, in one pool of reproductive tract of laying hens, it was possible to detect subtype B metapneumovirus. NDV was not detected in any sample.

In areas where there are many poultry farms, it is virtually impossible to keep chickens free of IBV. As the virus spread readily, vaccination is commonly practiced, (Cavanagh, 2007; Jackwood, 2012). In quail, despite the introduction of a vaccine program in some farms, using the only serotype permitted in Brazil as live attenuated vaccines (Massachusetts), *Avian coronavirus* IBV continued to be reported, as well as in chickens (Villarreal et al., 2010; Chacón et al., 2011; Balestrin et al., 2014).

The RdRp tree (Fig. 1) indicated that two quail strains (Farm3), one from the original sample of enteric content (EC) (Quail14/EC/Farm3) and the other from the cloning product of female reproductive tract (FRT) (Quail13/FRT clone5/Farm3) clustered with other gammacoronaviruses; whereas four clones, two from FRT of farm 3 (Quail13/FRTclone4/Farm3 and Quail13/FRTclone5/Farm6) and two from trachea (T) of farm 2 (Quail12/Tclone1/Farm2 and Quail12/Tclone2/Farm2) segregated with other deltacoronaviruses, showing that quail are susceptible for both genera of coronavirus.

Mean RdRp nucleotide identities of the quail strains Quail14/EC/Farm3 and Quail13/FRTclone5/Farm3 were 94% with the other gammacoronaviuses and strains Quail13/FRTclone4/Farm3, Quail13/FRTclone5/Farm6, Quail12/Tclone1/Farm2 and Quail12/Tclone2/Farm2) were 87% between other *Deltacoronavirus*.

Analysis of the S1 subunit (Fig. 2) revealed the circulation, in the same farm (Quail–Layers Farm2), of two lineages in the Brazil type of Avian coronavirus (one in quail and one in chickens), in agreement with the fact that IBV-types other than the archetypical ones are widespread in Brazil (Villarreal et al., 2010; Chacon et al., 2011; Balestrin et al., 2014). Moreover, genotyping results showed that all the IBV detected in samples from quail and layers were different from genotypes Massachusetts, D274, and 4/91.

The simultaneous detection of *Gamma and Deltacoronavirus* in the same flock (Quail Farm3) indicates that these viruses might co-infect quail. Co-infection of two or more coronaviruses in different birds within a region might not be unexpected, as this has been observed by Capua et al. (1999) with IBV in chickens and co-infection by both the gamma and delta groups were reported in ducks (Chu et al., 2011).

Further efforts on full-genome sequencing using next generation sequencing (NGS) of the quail coronaviruses detected herein will be carried out by the authors in order to better elucidate the origin of the *Deltacoronavirus* RdRp genes, providing valuable insight into the mechanisms behind coronaviral evolutionary biology and contributing to better control measures that prevent vaccine failures (Jackwood et al., 2012).

Although the IBV strains detected in this study could not be isolated in embryonated chicken eggs, sequence homology analysis of the sequenced PCR products obtained both in the replicase region and in S1 subunit, are evidence that viral RNA is present in the surveyed birds (De Wit, 2000).

In vivo pathogenicity assays using a quail Avian coronavirus still need to be carried out, but the results presented herein provide advances on previous studies on quail coronaviruses as it has been shown that: (a.) quail avian coronaviruses share spike genes identical to chicken IBVs spike genes and (b.) vaccination against IBV with the Massachusetts strain does not provide a sufficient control of IB in quail when Brazil type filed strains, are involved, similarly to what happens in chickens.

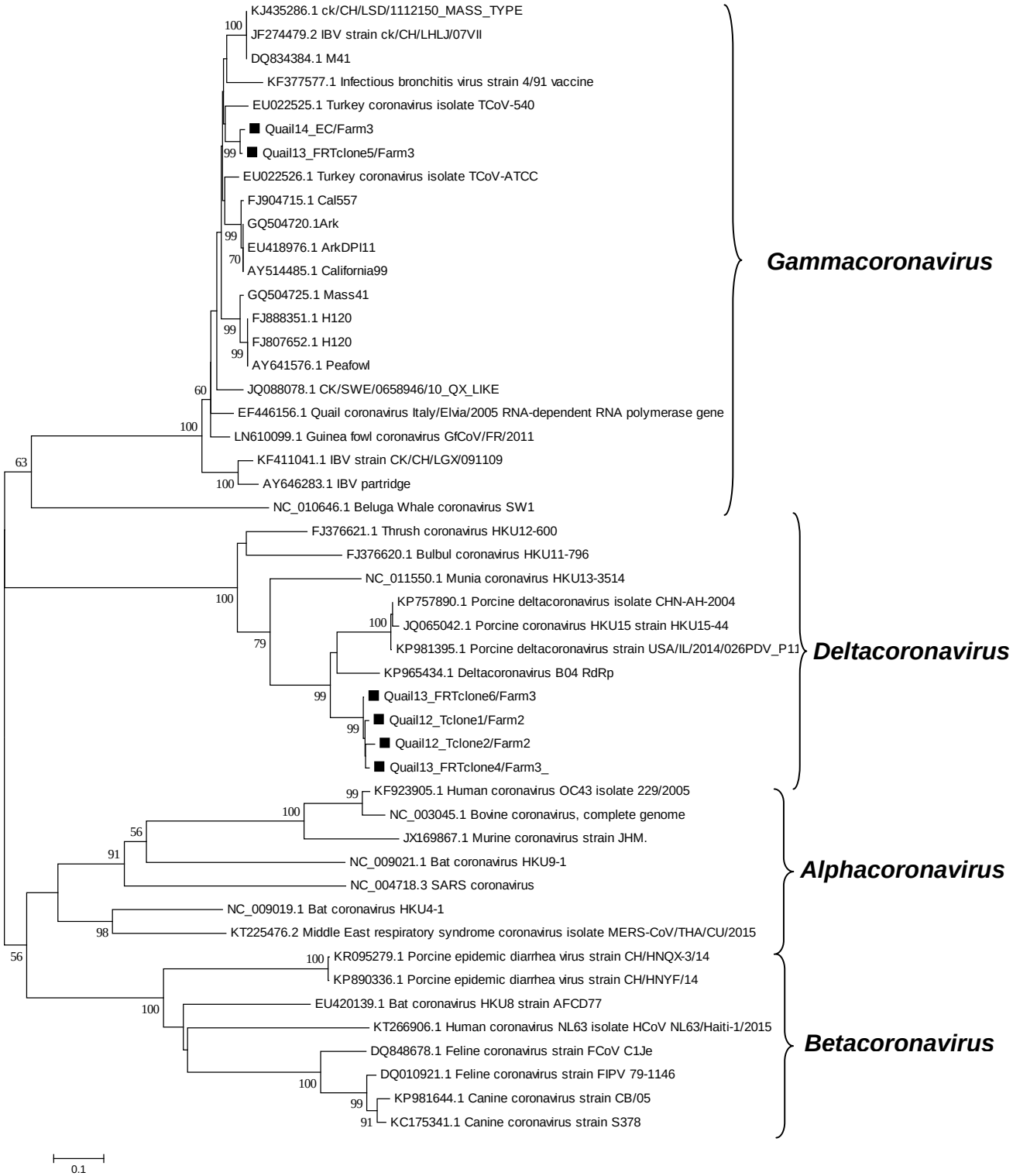
KJ435286.1 ck/CH/LSD/1112150_MASS_TYPE
JF274479.2 IBV strain ck/CH/LHLJ/07VII
DQ834384.1 M41
KF377577.1 Infectious bronchitis virus strain 4/91 vaccine
EU022525.1 Turkey coronavirus isolate TCoV-540
Quail14_EC/Farm3
Quail13_FRTclone5/Farm3
EU022526.1 Turkey coronavirus isolate TCoV-ATCC
FJ904715.1 Cal557
GQ504720.1Ark
EU418976.1 ArkDPI11
AY514485.1 California99
GQ504725.1 Mass41
FJ888351.1 H120
FJ807652.1 H120
AY641576.1 Peafowl
JQ088078.1 CK/SWE/0658946/10_QX_LIKE
EF446156.1 Quail coronavirus Italy/Elvia/2005 RNA-dependent RNA polymerase gene
LN610099.1 Guinea fowl coronavirus GfCoV/FR/2011
KF411041.1 IBV strain CK/CH/LGX/091109
AY646283.1 IBV partridge
NC_010646.1 Beluga Whale coronavirus SW1
Gammacoronavirus
FJ376621.1 Thrush coronavirus HKU12-600
FJ376620.1 Bulbul coronavirus HKU11-796
NC_011550.1 Munia coronavirus HKU13-3514
KP757890.1 Porcine deltacoronavirus isolate CHN-AH-2004
JQ065042.1 Porcine coronavirus HKU15 strain HKU15-44
KP981395.1 Porcine deltacoronavirus strain USA/IL/2014/026PDV_P11
KP965434.1 Deltacoronavirus B04 RdRp
Quail13_FRTclone6/Farm3
Quail12_Tclone1/Farm2
Quail12_Tclone2/Farm2
Quail13_FRTclone4/Farm3_
Deltacoronavirus
KF923905.1 Human coronavirus OC43 isolate 229/2005
NC_003045.1 Bovine coronavirus, complete genome
JX169867.1 Murine coronavirus strain JHM.
NC_009021.1 Bat coronavirus HKU9-1
NC_004718.3 SARS coronavirus
NC_009019.1 Bat coronavirus HKU4-1
KT225476.2 Middle East respiratory syndrome coronavirus isolate MERS-CoV/THA/CU/2015
Alphacoronavirus
KR095279.1 Porcine epidemic diarrhea virus strain CH/HNQX-3/14
KP890336.1 Porcine epidemic diarrhea virus strain CH/HNYF/14
EU420139.1 Bat coronavirus HKU8 strain AFCD77
KT266906.1 Human coronavirus NL63 isolate HCoV NL63/Haiti-1/2015
DQ848678.1 Feline coronavirus strain FCoV C1Je
DQ010921.1 Feline coronavirus strain FIPV 79-1146
KP981644.1 Canine coronavirus strain CB/05
KC175341.1 Canine coronavirus strain S378
Betacoronavirus
0.1

Figure 1. Phylogenetic distance tree with the neighbor-joining algorithm and MCL method for nucleotides of the RdRp gene (nt positions 16,179-16,543), showing the classic serotypes/ genotypes (with Genbank accessing numbers) of the genera *Alpha, Beta, Gamma* and *Deltacoronavirus* and strains included in this study (square for quail). 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.

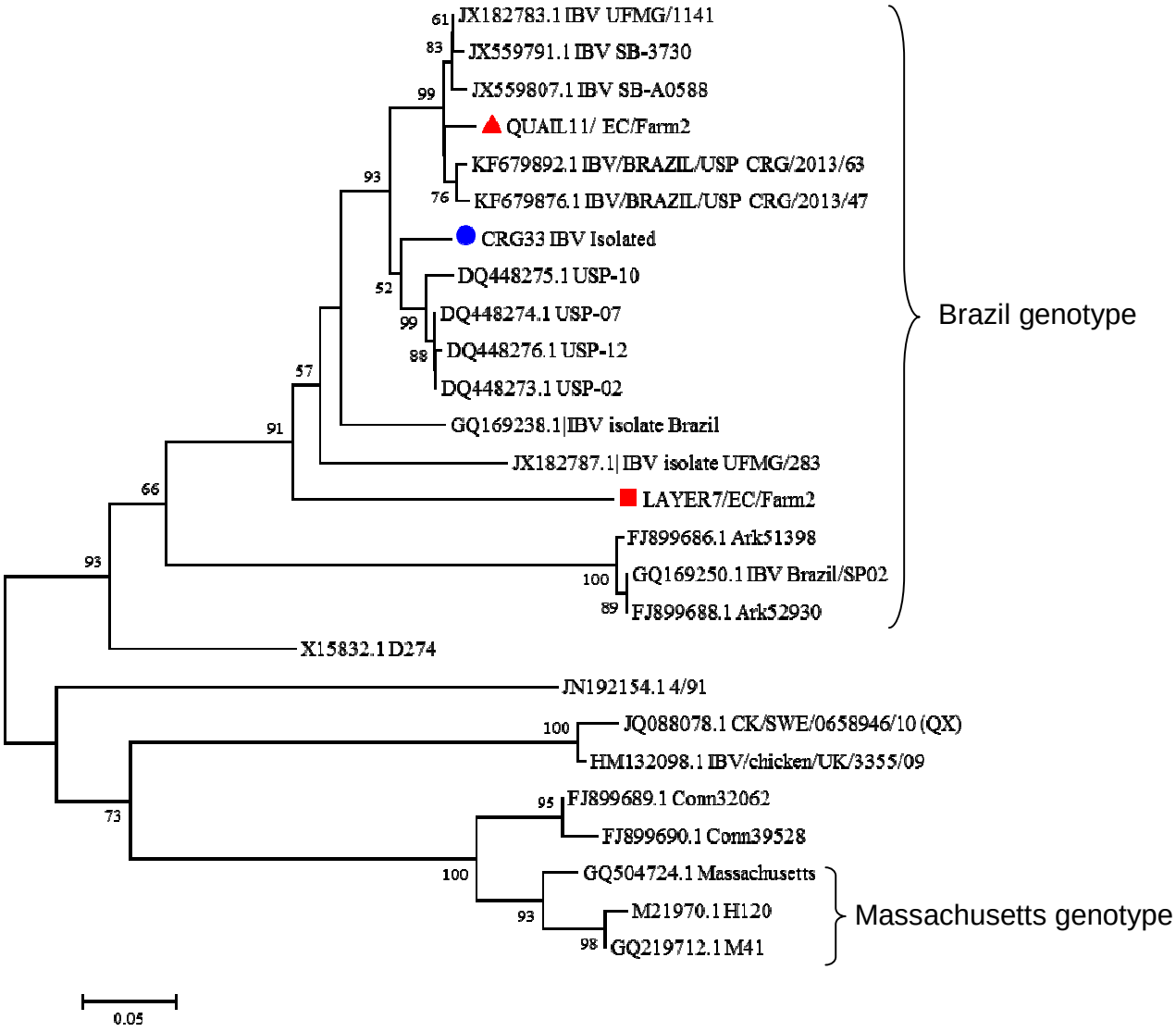

Figure 2. Phylogenetic distance tree with the neighbor-joining algorithm and MCL method for nucleotides of the spike gene S1 subunit (nt positions 28-477) showing classic serotypes/genotypes (with the access number from Genbank) and sequences from this study (the square represents a strain from a layer hen, the triangle a quails strain and the circle the CRG strain from a poultry flock and previously isolated in the laboratory in SPF eggs). 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.

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4 GAMMA AND DELTACORONAVIRUSES IN QUAIL FROM NORTHERN ITALY

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ABSTRACT

On the view of the restricted knowledge on the diversity of coronaviruses in poultry other than chicken, this study aimed to investigate the molecular diversity of coronaviruses in quail, pheasant and partridge from two regions of Northern Italy. For these porpoise, pools of tracheal and cloacal swabs and intestine/enteric content from European quail (*Coturnix coturnix*), pheasants (*Phasianus colchicus*) and partridge (*Perdix perdix)* flocks, respectively, with or without enteric signs, were collected during 2015. An *Avian coronavirus* was detected on unvaccinated IBV quail farms and vaccinated pheasant samples with an IBV Massachusetts serotype. *Avian metapneumovirus* and Newcastle disease virus was not found in any quail, pheasant or partridge sample. 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 analyses showed the susceptibility of quail to Deltacoronaviruses, suggesting that quail and pheasant avian coronaviruses share spike genes identical to chicken IBV spike genes and quail might host coronaviruses with RdRp gene related to *Deltacoronavirus.*

KEY WORDS *Gammacoronavirus, Deltacoronavirus,* quail, pheasant, IBV.

Abbreviations: IBV = Avian infectious bronchitis virus, IB = Infectious bronchitis, aMPV = Avian metapneumovirus, NDV = Newcastle disease virus, DEPC = diethyl pyrocarbonate, PBS= Phosphate buffered saline PHRED = Phil's Read Editor, QCoV = Quail coronavirus, *RdRp* = RNA- dependent RNA polymerase, RT-PCR = reverse transcription-polymerase chain reaction, S-gene = spike gene, UTR = untranslated region, v/v = volume/volume

INTRODUCTION

Coronaviruses (CoV), positive-sense single stranded RNA viruses (*Nidovirales: Coronaviridae*: *Coronavirinae*) are currently classified in four genera. *Alphacoronavirus (α-CoV)* and *Betacoronavirus* (*β-CoV)* infect many mammalian species ranging from bats to humans (Chan, et al. 2013) while *Gammacoronavirus* (*γ-CoV*) and *Deltacoronavirus* (*δ-CoV*) largely establish in birds and to a slightly degree in mammals (Woo et al., 2010; WOO et al., 2012; ICTV, 2014; Fehr & Perlman, 2015).

Avian coronavirus (Avian infectious bronchitis virus, IBV in chickens), the causative agent of avian infectious bronchitis (IB), is a *Gammacoronavirus* that can replicate on every epithelial surface, causing poultry diseases related to kidneys, reproductive and respiratory systems and enteritis (Cavanagh, 2003; Cavanagh, 2005; Villarreal et al., 2007). IBV and IBV-like strains can be detected in both gallinaceous and non-gallinaceous birds, asymptomatically in some cases (Cavanagh, 2005), such as geese, ducks, pigeons, pheasant (Cavanagh et al., 2002) and quail (Circella et al., 2007; Torres et al., 2013) and this might suggest that these species would act as vectors of IBV and spread of IBV strains over the world (De Witt et al., 2011).

Other CoVs in asymptomatically wild birds, 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).

On the view of the restricted knowledge on the diversity of coronaviruses in poultry other than chicken, this study aimed to investigate the molecular diversity of coronaviruses in quail, pheasant and partridge from Northern Italy.

MATERIAL AND METHODS

Farms. Birds were sampled in 10 farms from 17 quail, 8 pheasant and 2 partridge flocks, resulting in a total of 39 samples of quail, 8 of pheasants and 2 of partridges in 2015. Four quail-only farms (identified as F3 to F5) and one quail/broiler farm (identified as F1) were sampled at the beginning, middle and end of the production cycle in the Verona and Vicenza province (Veneto region) and Ravenna and Forlì- Cesena province (Emilia Romagna region), respectively.

Farms of only pheasant and partridge (identified as F6 to F9 and F10, respectively) were also sampled by field veterinarians in Verona providence, Veneto region and send to the Virology Laboratory of Padova University (MAPS-UNIPD). All farms were located in Northern Italy and had history of birds with and without enteric signs such as diarrhea or feaces with mucous content.

In the study farms, quail were not vaccinated against IBV; nonetheless, vaccination against IBV using attenuated Massachusetts (Mass) serotype was applied at the quail breeders of farm five (F5). Broilers from the quail-broiler farm had been vaccinated against IBV at hatchery using attenuated Mass or 793B serotype. Regarding to pheasants, farm six (F6) vaccinated at one day old with attenuated IBV Mass serotype, while for partridge no vaccination was reported. All birds used in this study were not vaccinated against *Avian metapneumovirus* (aMPV) and information about vaccination against Newcastle disease virus (NDV) for all the species was not available.

Sample collection. Samples were collected from birds with or without clinical signs as pools of cloacal and tracheal swabs (10 birds /flock) or complete intestine/enteric content (5 birds/flock). Additionally, 10 tracheal and cloacal swabs per quail flock were collected for viral isolation and transported in 5 ml of MEM SF 10X Pen-Strept™ (Minimum Essencial Medium with streptomycin 10,000 µg/ml and penicillin G sodium 10,000 units/ml),. All samples were stored at -80° C until processing.

Positive and negatives controls. The IBV Massachusetts and QX vaccine strains (NOBILIS™ IB Ma5 and NOBILIS™ IB Primo QX, MSD Animal Health, Boxmeer, The Netherlands) and aMPV strain vaccine K (subtype A) and VCO3/50 (subtype B), were used as positive controls of the RT-PCR and RTq-PCR, respectively. As negative control RNAse and DNAse-free water was used.

RNA extraction. Pools of tracheal and cloacal swabs were prepared in 2 mL sterile PBS 10% and pools of intestine/enteric content were prepared as 50% (v/v) suspensions in PBS 10% and clarified at 5000 x g for 15 min at 10°C. Total RNA was extracted from 200µl of the supernatants and from samples placed directly in PBS 10% with High Pure

RNA Isolation kit™ (Roche Diagnostics Italy) according to the manufacturer's instructions.

One step nested RT- PCR for *Avian coronavirus* and NDV screening. Each sample was screened for *Avian coronavirus* and NDV as described by Cavanagh et al. (2002) and Kho et al. (2000), targeting the 3'-untranslated region (UTR) and F gene, respectively, using SuperScript™ III RT/ Platinum™ Taq Mix (Invitrogen, Carlsbad, CA, EUA) as per manufacturer's instructions, with amplicons of 179 and 532 base pairs (bp), respectively.

Quantitative real-time RT-PCR (qRT-PCR) with a molecular beacon probe assay for aMPV screening. Each sample was screened for aMPV as described by Cecchinato et al., (2013), targeting the SH gene of subtypes A and B. All reactions were carried out on LightCycler 480™ (Roche Diagnostic, Marnes La Coquette, France) using a SuperScript™ III qRT/ Platinum™ Taq DNA Polymerase (Invitrogen, Carlsbad, CA, EUA) as manufacturer's instructions.

RNA-dependent RNA polymerase (*RdRp*) and Spike gene analysis. 3'UTR- *Avian coronavirus* positive samples were then tested by a pan-coronavirus RT-PCR targeting the RNA- dependent RNA polymerase (*RdRp*) gene as described by Chu et al. (2011) (440bp amplicon) and also by a typing S-gene targeted multiplex RT-PCR for 793/B, D274 and Massachusetts types (Cavanagh et al., 1999) and a generic S gene RT-PCR described by Worthington et al. (2008) (390bp amplicon), using SuperScript™ III RT/ Platinum™ Taq Mix (Invitrogen, Carlsbad, CA, EUA) as per manufacturer's instructions.

All RT-PCR products bands were analyzed by electrophoresis in 2% (w/v) agarose in 1×TBE buffer, stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, EUA) and visualized by ultraviolet transillumination.

Amplicons were sent to bi-directional DNA sequencing in Macrogen (Macrogen Europe). 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 Figure 1 and 2) with Clustal/W multiple alignment using the same software.
Nucleotide (Neighbor-joining, Maximum Composite Likelihood model, 1,000 bootstrap replications) and amino acids trees (Neighbor-Joining, Poisson correction, 1,000 bootstrap replications) for S gene and *RdRp* were built using MEGA 5.2.1 (Tamura et al., 2011).

Virus isolation and propagation. Three quail coronavirus-positive swab pools (one cloacal and two tracheal) and negative for aMPV and NDV, were used for virus isolation in chicken tracheal organ culture (TOC) as described by Cook et al (1976). Briefly, each swab was homogenized in Eagle's MEM SF 10X Pen-Strept™, clarified at 4 °C at 1,000 g for 10 min, filtered through a 0.45µm pore membranes and 0.1 ml of each filtrate was inoculated into 10 trachea rings previously drained. After one hour of adsorption at 38°C, 0.5 ml of MEM SF™ (Invitrogen, Carlsbad, CA, EUA) heated at 38°C were added. The inoculated TOCs were incubated at 38°C and examined microscopically daily to score the level of ciliary activity using scale from 0 (100% of cilia beating) to 4 (0% of cilia beating). On day 4 post-inoculation (PO), maintenance MEM SF™ (Invitrogen, Carlsbad, CA, EUA) were harvested as pools from 5 of 10 TOCS and inoculated into 10 new TOCS. The remaining five TOCS were observed during 6 more days to score the ciliary activity. Virus isolation was also monitored with the screening PCRs for 3'UTR described above using the maintenance MEM SF™ (Invitrogen, Carlsbad, CA, EUA) as samples.

RESULTS

***Avian coronavirus*, aMPV and NDV screening**. Of the 10 farms analyzed, 15 flocks (9 of quail and 6 of pheasant) from 7 farms (F1, F2, F4, F5, F6, F7 and F8) were positive for *Avian coronavirus*. Viral RNA from all studied flocks was detected in 32.6% pools (10 quail and 6 pheasant/49 total pools) but not in any partridge sample. Moreover, aMPV and NCD were not detected in any sample.

RNA-dependent RNA polymerase (*RdRp*) and Spike gene sequence analysis. The multiplex typing PCR revealed that all strains of IBV detected in samples from quail and pheasants belong to the 793B and Massachusetts genotypes, respectively.

Sequences of 394 nucleotides (RdRp) and 347 nucleotides (S) from quail samples of cloacal (F1 and 4) and tracheal (F1 and 2) swabs and samples of intestine/enteric content belonged to pheasants (F6) were obtained for phylogenetic analyses (Table 1). The phylogenetic trees (Fig. 1) for the RdRp gene indicates that quail strains from this study (Quail01/CS/Farm1, Quail06/CS/Farm1, Quail11/TS/Farm2, Quail21/CS/Farm1, Quail22/TS/Farm1, Quail30/CS/Farm4, Quail32/CS/Farm4 and Quail38/CS/Farm1 clustered with a strain isolated from a Brazilian *Amazona vinacea* (Durães-Carvalho et al., 2015, Genbank KP965434) in the δ-CoV genus. Moreover the analyses revealed different viral populations in the quail strains as three subclusters were formed, with a mean nucleotide identity of 95.89%.

Three pheasant strains from this study (Pheasant01/EC/Farm6, Pheasant02/EC/Farm6, Pheasant03/EC/Farm6) clustered in the *Gammacoronavirus* genus (Fig. 1), with 100% identity amongst each other.

Mean RdRp nucleotide identities of the quail strains was 54.45% when compared to strain from pheasants, 54,5% with the other gammacoronaviuses in Fig. 1 and 92.5% and 86% between quail- *Deltacoronavirus* B04 genotype and Porcine *Deltacoronavirus*, respectively. The S1 subunit phylogenetic tree (Fig. 2) showed the susceptibility of quail to the 793B IBV archetype (mean nucleotide identity of 97%) and in two of those lineages, one from quail-only (F1) and the other from quail-broiler (F2) there was a mean nucleotide identity of 99.4% with a commercial IBV vaccine used at broiler´s hatchery based on the 793B type. The three pheasant strains clustered with Massachusetts type with a 99% nucleotide identity.

Virus isolation. After two passages, TOCs scored 0 (100% of cilia beating), resulting in negative isolation and confirmed by RT-PCR directed to the 3'UTR.

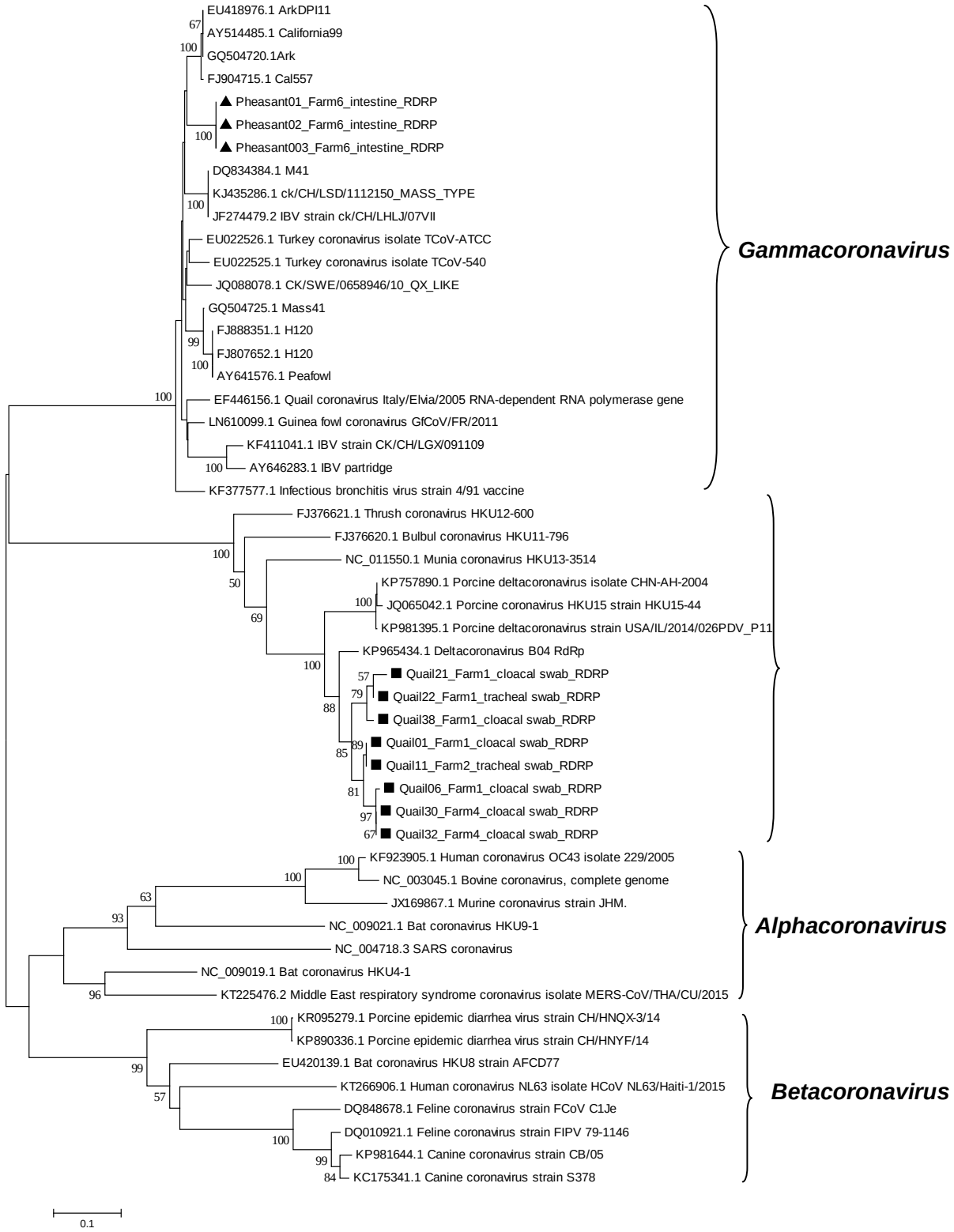
EU418976.1 ArkDPI11
AY514485.1 California99
GQ504720.1Ark
FJ904715.1 Cal557
Pheasant01_Farm6_intestine_RDRP
Pheasant02_Farm6_intestine_RDRP
Pheasant003_Farm6_intestine_RDRP
DQ834384.1 M41
KJ435286.1 ck/CH/LSD/1112150_MASS_TYPE
JF274479.2 IBV strain ck/CH/LHLJ/07VII
EU022526.1 Turkey coronavirus isolate TCoV-ATCC
EU022525.1 Turkey coronavirus isolate TCoV-540
JQ088078.1 CK/SWE/0658946/10_QX_LIKE
GQ504725.1 Mass41
FJ888351.1 H120
FJ807652.1 H120
AY641576.1 Peafowl
EF446156.1 Quail coronavirus Italy/Elvia/2005 RNA-dependent RNA polymerase gene
LN610099.1 Guinea fowl coronavirus GfCoV/FR/2011
KF411041.1 IBV strain CK/CH/LGX/091109
AY646283.1 IBV partridge
KF377577.1 Infectious bronchitis virus strain 4/91 vaccine
Gammacoronavirus
FJ376621.1 Thrush coronavirus HKU12-600
FJ376620.1 Bulbul coronavirus HKU11-796
NC_011550.1 Munia coronavirus HKU13-3514
KP757890.1 Porcine deltacoronavirus isolate CHN-AH-2004
JQ065042.1 Porcine coronavirus HKU15 strain HKU15-44
KP981395.1 Porcine deltacoronavirus strain USA/IL/2014/026PDV_P11
KP965434.1 Deltacoronavirus B04 RdRp
Quail21_Farm1_cloacal swab_RDRP
Quail22_Farm1_tracheal swab_RDRP
Quail38_Farm1_cloacal swab_RDRP
Quail01_Farm1_cloacal swab_RDRP
Quail11_Farm2_tracheal swab_RDRP
Quail06_Farm1_cloacal swab_RDRP
Quail30_Farm4_cloacal swab_RDRP
Quail32_Farm4_cloacal swab_RDRP
KF923905.1 Human coronavirus OC43 isolate 229/2005
NC_003045.1 Bovine coronavirus, complete genome
JX169867.1 Murine coronavirus strain JHM.
NC_009021.1 Bat coronavirus HKU9-1
NC_004718.3 SARS coronavirus
NC_009019.1 Bat coronavirus HKU4-1
KT225476.2 Middle East respiratory syndrome coronavirus isolate MERS-CoV/THA/CU/2015
Alphacoronavirus
KR095279.1 Porcine epidemic diarrhea virus strain CH/HNQX-3/14
KP890336.1 Porcine epidemic diarrhea virus strain CH/HNYF/14
EU420139.1 Bat coronavirus HKU8 strain AFCD77
KT266906.1 Human coronavirus NL63 isolate HCoV NL63/Haiti-1/2015
DQ848678.1 Feline coronavirus strain FCoV C1Je
DQ010921.1 Feline coronavirus strain FIPV 79-1146
KP981644.1 Canine coronavirus strain CB/05
KC175341.1 Canine coronavirus strain S378
Betacoronavirus
0.1

Figure 1. Phylogenetic distance tree with the neighbor-joining algorithm and MCL method for nucleotides of the RdRp gene (nt position 16,179-16,543), showing the classic serotypes/ genotypes (with Genbank accessing numbers) of the genera *Alpha, Beta, Gamma* and *Deltacoronavirus* and strains included in this study (square for quail and triangle for pheasant, respectively). 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.

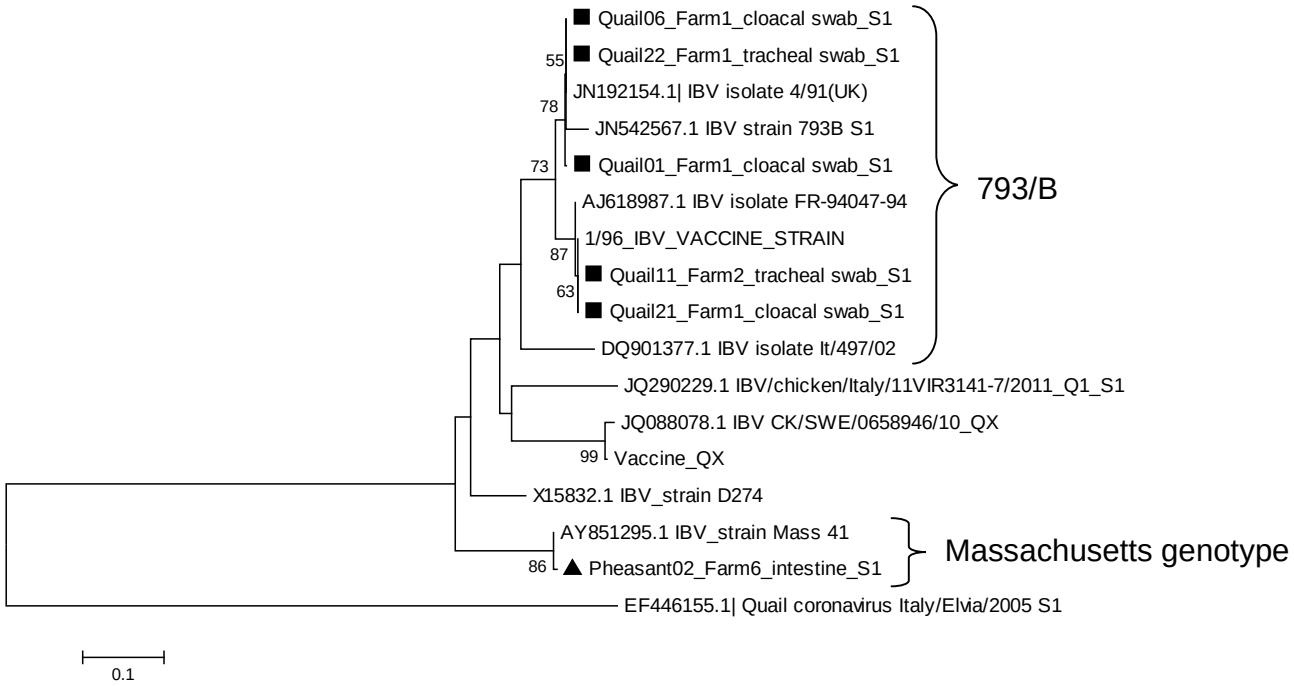

Figure 2. Phylogenetic distance tree with the neighbor-joining algorithm and MCL method for nucleotides of the S gene (nt position 726-1,073), showing the classic serotypes/ genotypes (with Genbank accessing numbers) and strains included in this study (square for quail and triangle for pheasant, respectively). 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.

DISCUSSION

The molecular diversity of coronaviruses in quail, pheasants and partridge from two regions of Northern Italy was evaluated, allowing the detection of *Avian coronavirus* IBV strains related to the 793B and Massachusetts types in quail and pheasants, respectively, and quail were shown as hosts to Deltacoronaviruses.

The phylogenetic analysis of the S1 subunit (Fig 2) revealed the susceptibility of quail to 793/B IBV archetype as all ready reported in chickens of Italy (Capua et al., 1999), UK (Jones et al., 2005) and other western European countries chickens (Krapez, et al. 2011; Fellahi et al., 2015). Moreover, two quail strains (Quail11/TS/Farm2 and Quail21/CS/Farm1), one for quail only farm and other from quail-broilers farm, clustered with an IBV 793B vaccine strain widespread applied in Italy, suggesting a vaccine origin for these strains.

Regarding pheasants, all strains clustered with Massachusetts genotype; considering that these birds had been vaccinated with a Massachusetts live vaccine, this also suggests a vaccine origin for this detection, as IBV vaccines might be detected for weeks after vaccination (Cavanagh et al., 1999).

The RdRp gene tree (Fig 1.) showed the susceptibility of quail to δ-CoV as all quail lineages obtained in this study segregated with an δ-CoV isolated from Brazilian wild birds (*Amazona vinacea*) and are related to other δ-CoV viruses isolated from swine, previously unreported in Italy, showing that quail are also susceptible to this genus of coronavirus.

It's noteworthy that no IBV-like RdRp gene has been detected in the quail samples studied herein. The previously reported Quail coronavirus (QCoV) (CIRCELLA et al., 2007) based on the RdRp gene, showed a 16-18% identity with IBV, suggesting that specifically QCoV was not a variant IBV and the amino acid sequence of the S1 portion QCoV had 79-81% identity with TCoV strain, which might mean that the QCoV is similar to the turkey coronavirus.

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-Delta co-infection and a recombination event might be hypothesized. Nonetheless, between-genera recombination for

coronaviruses, though initially considered but latter disregarded as a source of the SARS coronavirus, seem to be unlike in nature (Thor et al, 2011, Woo et al., 2010).
Further efforts on full-genome sequencing using next generation sequencing (NGS) of the quail coronaviruses detected herein will be carried out by the authors in order to better elucidate the origin of the *Deltacoronavirus* RdRp genes.

Although the coronaviruses strains detected in this study could not be isolated in TOC, sequence homology analyses of the sequenced PCR products obtained both in the replicase region and in S1 subunit suggests that coronavirus RNA was present in the surveyed birds, though no affirmations on the infectivity of these strains can be made based on these PCRs (DE WIT, 2000).

Isolation and i*n vivo* pathogenicity assays using a quail coronavirus still need to be carried out, but the results presented herein allow to conclude that (a.) quail and pheasant avian coronaviruses share spike genes identical to chicken IBVs spike genes and (b.) quail might host coronaviruses with RdRp gene related to *Deltacoronavirus.*

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Table 1. Strains include in the analysis of the RNA- dependent RNA polymerase (*RdRp*) gene and S1 subunit of the S gene according, specie (quail or pheasant), clinical signs at the sampling time and type of sample.

Strain	Host	Signs	Sample	Gene
Quail01/CS/Farm1	Quail	N/S	Cloacal swab	RDRP
Quail01/CS/Farm1	Quail	N/S	Cloacal swab	S
Quail06/CS/Farm1	Quail	N/S	Cloacal swab	RDRP
Quail06/CS/Farm1	Quail	N/S	Cloacal swab	S
Quail11/TS/Farm2	Quail	Ent	Tracheal swab	RDRP
Quail11/TS/Farm2	Quail	Ent	Tracheal swab	S
Quail21/CS/Farm1	Quail	N/S	Cloacal swab	RDRP
Quail21/CS/Farm1	Quail	N/S	Cloacal swab	S
Quail22/TS/Farm1	Quail	N/S	Tracheal swab	RDRP
Quail22 TS/Farm1	Quail	N/S	Tracheal swab	S
Quail30/CS/Farm4	Quail	Ent	Cloacal swab	RDRP
Quail32/CS/Farm4	Quail	Ent	Cloacal swab	RDRP
Quail38/CS/Farm1	Quail	Ent	Cloacal swab	RDRP
Pheasant01/EC/Farm6	Pheasant	Ent	Enteric content	RDRP
Pheasant02/EC/Farm6	Pheasant	Ent	Enteric content	RDRP
Pheasant03/EC/Farm6	Pheasant	Ent	Enteric content	RDRP
Pheasant02/INT/Farm6	Pheasant	Ent	Intestine	S

Ent= Enteric

N/S= Not signs

5 FINAL REMARKS

This thesis describes the epidemiological situation of quail farms in Brazil pre- and post-vaccination against IBV and in Italy in which no IBV vaccination schedule was applied in quail, in order to expand previous studies on quail coronaviruses.

To this end, in 2013 samples were collected in São Paulo, southeastern Brazil, where some farmers have started vaccination of quail against IBV, using the Massachusetts serotype vaccine, after the surveillance conducted in 2009-2010. Pools of tracheas, lungs, reproductive system, kidneys and enteric content were collected from quail and layers housed in the same farms and quail exclusive farm. The animals showed clinical signs similar to those seen in infectious bronchitis (IBV).

Regarding the Italian farms, samples of the northern region (Emilia Romagna and Veneto) were collected as pools of trachea and cloacal swabs form quail co-housed with broilers on interspersed production cycles that means, in one cycle only quail were housed and on the successive cycle, quail and broilers were housed on the same farm, and quail-only farms. Birds showed clinical signs such as diarrhea or feaces with mucous content.

In this investigation, *Gammacoronavirus*, from symptomatic/asymptomatic birds, was detected in 88% (8/9) of the studying farms, in agreement with previously studies conducted by Torres et al. (2013) where *Avian coronavirus* closely related to infectious bronchitis virus has been identified in the Bastos region-Brazil and also with Circella et al. (2007) who detected, in a quail farm located in southern Italy, the presence of CoV; nevertheless, Circella et al. (2007) proposed that this coronavirus belonged to a new avian CoV specie denominated Quail coronavirus (QCoV).

Based on the DNA analyses of the S1 subunit, all *Avian coronavirus (Gammacoronavirus)* quail strains are related to IBV archetypes widespread in poultry farms in Brazil and Italy, as Brazil type (BR) and 793B, respectively, showing the susceptibility of quail to these archetypes.

In Brazilian quail, despite the introduction of a vaccine program in some farms, using the only serotype permitted in Brazil as live attenuated vaccines (Massachusetts), *Avian coronavirus* IBV continued to be reported, as well as in chickens (VILLARREAL et al., 2010; CHACÓN et al., 2011; BALESTRIN et al., 2014). Otherwise, Italian quail farms analyzed in this study were not vaccinated against IBV, however, two quail strains, one for quail only farm and other from quail-broilers farm, clustered with an IBV 793B vaccine strain (used in broilers study farm, at hatchery) widespread applied in Italy, suggesting a vaccine origin for these strains.

The RdRp gene analyses showed the susceptibility of Brazilian and Italian quail to *Deltacoronavirus* as all quail lineages obtained in this study segregated with other Deltacoronaviruses being some lineages previously reported in Brazilian wild birds (DURÃES-CARVALHO et al., 2015) but unreported in Italy. Even more, simultaneous detection of *Gamma and Deltacoronavirus* in two Brazilian quail sequences from the same flock (Quail Farm3) indicates that these viruses might co-infect quail, as already reported in wild birds (CHU et al., 2011; DURÃES-CARVALHO et al., 2015; JORDAN et al., 2015).

Taking into account the discrepant taxonomy of the quail coronaviruses detected herein regarding the RdRp (*Gammacoronavirus* or/and *Deltacoronavirus)* and the spike genes (*Gammacoronavirus*) either a Gamma-Delta co-infection or a recombination event, in the Italian scenario, might be hypothesized. Co-infection by both the gamma and delta groups were reported in ducks (CHU et al., 2011); nonetheless, between-genera recombination for coronaviruses, though initially considered but latter disregarded as a source of the SARS coronavirus, seem to be unlike in nature (THOR et al, 2011, WOO et al., 2010).

Further efforts on full-genome sequencing using next generation sequencing of the quail coronaviruses detected herein will be carried out by the authors in order to better elucidate the origin of the *Deltacoronavirus* RdRp genes, providing valuable insight into the mechanisms behind coronaviral evolutionary biology and contributing to better control measures that prevent vaccine failures (JACKWOOD et al., 2012).

6 CONCLUSIONS

a. Quail Avian coronavirus (*Gammacoronavirus*) strains share spike genes identical to chicken IBVs spike genes, both in Brazil and Italy and are also susceptible to *Deltacoronavirus.*

b. Vaccination against IBV with the Massachusetts strain has not provided a sufficient control of IB in Brazilian quail under field conditions when the *Brazil* type field strains are involved, similarly to what happens in chicken.

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