Phylogenetic and molecular characterization of coronavirus affecting species of bovine and birds in Cuba

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ABSTRACT

Avian infectious bronchitis virus (IBV) and bovine coronavirus (BCoV) are pathogens of veterinary importance that affect birds and bovine in Cuba; however, molecular characteristics and genetic diversity of these viruses are unknown. This study was aimed at determining the molecular characteristics and genetic diversity of both agents, based in the spike S gene. A molecular analysis was carried out from field strains of BCoV collected between 2009 and 2011 and phylogenetic studies were conducted using partial or complete S gene sequences as phylogenetic markers. Besides, studies of phylogenetic inference were carried out in S1 region of recent isolates of IBV. All Cuban bovine coronavirus sequences were located in a single cluster supported by 100 % bootstrap and 1.00 posterior probability values. The Cuban BCoV sequences were also clustered with the USA BCoV strains corresponding to the GenBank accession numbers EF424621 and EF424623, suggesting a common origin for these viruses. This phylogenetic cluster was also the only group of sequences in which no recombination events were detected. Of the 45 amino acid changes found in the Cuban strains, four were unique. On the other hand, two putative genotypes genetically different to the Massachusetts genotype H120 strain used in the Cuban vaccination program were found in the flocks assessed. In addition, a potential nephropathogenic IBV isolate was found by first time in Cuba. This research won the 2012 Award of the Cuban National Academy of Sciences.

Keywords: avian infectious bronchitis virus, bovine coronavirus, phylogenetic and molecular characterization

RESUMEN

Caracterización filogenética y molecular de Coronavirus que afectan a especies de bovinos y aves en Cuba. El virus de la bronquitis infecciosa aviar (IBV) y el coronavirus bovino (BCoV) son agentes patógenos de importancia veterinaria porque afectan a las aves y al ganado bovino en Cuba. Como se desconocen sus características moleculares y diversidad genética, el objetivo de esta investigación fue determinarlas en ambos agentes, a partir del gen de la espícula S. Se analizaron cepas de campo de BCoV, recolectadas entre los años 2009 y 2011, y se compararon filogenéticamente según varios marcadores del gen S. Además se hicieron estudios de inferencia filogenética en una región de S1 de aislados recientes del IBV. Todas las cepas de BCoV cubanas se localizaron en un mismo grupo filogenético con un soporte estadístico del 100 % y valor de probabilidad posterior 1.00. Las secuencias de coronavirus bovino cubanas se agruparon con cepas de BCoV de EE.UU. con números de acceso en el GenBank de EF424621 y EF424623, que sugiere un origen común para estos virus. Este grupo filogenético fue el único en cuyas secuencias no se detectaron eventos de recombinación. De los 45 cambios de aminoácidos en las cepas cubanas, cuatro fueron únicos. En las granjas evaluadas se detectaron dos posibles genotipos genéticamente diferentes al genotipo Massachusetts de la cepa H120 usada en el programa de vacunación cubano. Además, se halló un potencial aislado de IBV nefropatogénico encontrado por primera vez en Cuba. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba del año 2012.

Palabras clave: virus de la bronquitis infecciosa aviar, coronavirus bovino, caracterización filogenética y molecular

Introduction

The *Coronavirus* family has been studied for more than 50 years like virus that infect different species of animals, including the human. Several of these viruses, as the bovine coronavirus (BCoV) and infectious bronchitis virus (IBV), are of veterinary importance due to the respiratory and gastrointestinal disorders that they produce in domestic animals and the continuous emergency of new species [1], groups [2] or variants.

In the case of IBV the continuous emergency and dissemination of different geno/serotypes have committed in the world the control of the disease caused

by this agent. Several reports have showed the continuous emergency of new variants caused by the evolution of field strains [3-4] or recombination events between heterologous strains classified in different genetic groups [5]. Therefore, identifying the diversity and molecular characteristics of the geno/serotypes that circulate in the populations is an indispensable task for controlling them. On the other hand, a better understanding of the molecular bases of BCoV evolution, tropism and virulence have been continuously demanded from the scientific community due to its economic relevance for cattle industry and its biological

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and antigenic similarities with another Betacoronavirus as the human coronavirus OC43 (HCoV OC43). Additionally, the BCoV shares biological pathogenic and pneumoenteric properties with species of Coronavirus related with the SARS (SARS-CoVs) [6].

In order to fulfill its primary objectives on behalf of its mission to "Preserve the animal and human health", the Center for animal Health (Censa, Cuba) required to incorporate all the genetic and molecular information on animal emergent pathogens. In this sense, it follows the purpose of developing diagnostic assays to detect the escape of new strains, tracing their origin and dissemination and to determine their potential virulence and pathogenicity. This research won the 2012 Award of the Cuban National Academy of Sciences.

The objectives of present work were: To determine the genetic diversity and the phylogenetic relationships among infectious bronchitis virus (IBV) isolates associated with respiratory disease from Cuban chicken flocks based on partial S1 phylogenetic marker; and to compare the amino acidic identities of the spike gene vaccine virus and the isolates circulating in the poultry farms of Cuba.

Additionally, regarding Bovine coronavirus (BCoV), the studies were intended to determine the genetic diversity and the phylogenetic relationships among different isolates of BCoV associated with similar diseases to WD and respiratory in the bovine livestock in Cuba. Also to establish the presence of markers related with tropism, pathogenicity and host range.

*R*esults

Molecular genetic diversity of infectious bronchitis virus in Cuba

Avian infectious bronchitis (IB) is a highly contagious viral disease of poultry characterized by respiratory signs, nephritis and reduced egg production [7]. This disease is caused by IBV, a member of the genus Gamma coronavirus, family *Coronaviridae*, order Nidovirales [8] which is considered a major pathogen in poultry production [9].

It has been reported that the S1 gene sequence comparison is a good predictor of cross-protection between different geno/serotypes of the virus in vaccinated populations [10]. Therefore, the knowledge of the sequences of this gene as well as the phylogenetic analysis of isolates that circulates in the populations of each country is an indispensable task of the diagnosis laboratories in order to face the control of this agent.

The current work constitutes the first genetic study of IBV in Cuba. Previous studies based on cross-neutralization showed the Mass genotype as the genotype that circulated in the Cuban chicken flocks [11]. The genetic analysis and the phylogenetic relationships among recent IBV Cuban isolates and reference IBV strains suggested that different variants of the virus are circulating among chicken flocks (Figure 1).

It was interesting the low degree of amino acid identity values found among the Mass genotype and two Cuban IBV recent isolates: Cuba/LaHabana/CB6/2009 and Cuba/La Habana/CB19/2009. This indicated a possible evasion to the host immune response due to a poor effectiveness of the vaccine based on
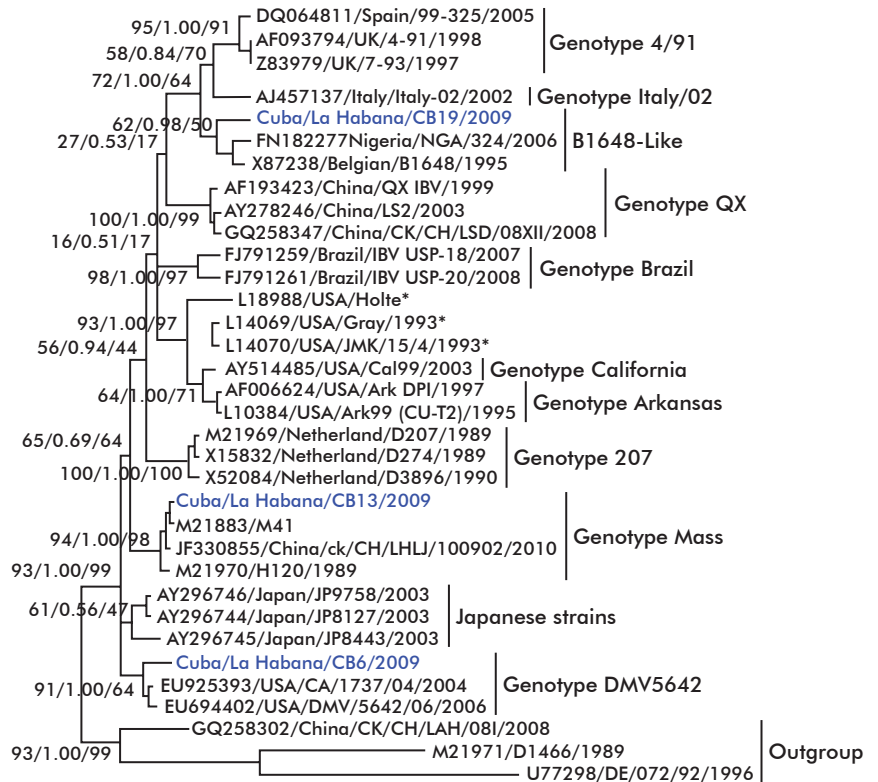

Figure 1. Phylogenetic tree of avian infectious bronchitis virus (IBV) genotypes. The Cuban IBV sequences are denoted in blue and are deposited at the EMBL/GenBank database under the Accession Numbers HE590762-HE590764. A partial S1 sequence (390 bases) phylogenetic marker previously proposed was carried out. After the alignment, phylogenetic relationships among IBV genotypes were analyzed using Neighbor-Joining (NJ) and Bayesian Inference (BI). Additionally, Maximum Likelihood (ML) tree was computed using the PHYML v3.0, confidence was estimated by 1000 bootstrap replicates. The topologies were tested by the Kishino and Hasegawa test (K-H) and the Shimodaira-Hasegawa test (S-H), using the PAMLv4.3 program. Numbers along the branches refer to the percentages of confidence in the ML, posterior probability in the BI and percentages of confidence in the NJ analyses.

the H120 strain, from the Mass genotype, applied in Cuban poultry flocks, against field isolates (Table).

Besides, the fact that the isolate Cuba/La Habana/CB19/2009 showed a close relationship with the nephropathogenic isolate B1648 could suggest that this Cuban isolate might be a nephropathogenic IBV isolate established in the field.

Genetic diversity and the possible origin of BCoV field strains in different regions of Cuba

BCoV was first identified in association with diarrhoea in newborn calves [12] and later associated with winter dysentery (WD) in adult cattle [13] and respiratory tract infections in calves and feedlot cattle [14]. Although the affected animals rarely die, coronavirus infection causes dramatic reductions in milk production in dairy herds and loss of body condition in both calves and adults [15], resulting in severe economic losses. Thus, BCoV is currently considered an important pathogen that causes enteric disease, often in combination with clinical respiratory signs.

The S glycoprotein is important for viral entry and pathogenesis, forms large petal-shaped spikes on the surface of the virion and is cleaved into S1 (N-terminus) and S2 (C terminus) subunits [16]. The S1 is the globular subunit and is responsible for virus binding to host-cell receptors [17], the induction of

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Table. Nucleotide and deduced amino acid identities of avian infectious bronchitis virus (IBV) hypervariable region of the S1 gene*

Genotype	No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	No.
									Nucleotide identity (%)										
Cuba/La Habana/CB6/2009	1		87.0	81.8	80.6	91.3	83.7	76.6	78.0	76.9	80.1	80.1	76.6	82.1	81.2	78.6	80.6	79.2	1
Cuba/La Habana/CB19/2009	2	70.7		79.2	87.8	80.9	80.0	77.8	80.6	78.9	79.5	84.1	78.6	79.5	84.4	80.1	81.8	79.8	2
Cuba/La Habana/CB13/2009	3	58.0	55.7		77.8	83.8	84.8	81.5	82.4	81.2	79.8	76.9	81.5	99.7	77.2	82.7	80.4	80.1	3
Belgian/B1648/1996	4	60.1	77.4	54.8		78.3	80.0	77.2	81.5	79.2	78.3	87.3	79.2	77.8	85.8	79.2	80.1	82.1	4
USA/DMV/5642/06	5	78.3	56.6	62.8	53.9		84.8	78.6	79.5	78.3	79.8	77.8	78.0	84.1	78.6	78.9	80.4	81.2	5
Japan/JP9758/2003	6	30.4	24.3	32.4	24.3	28.6		78.8	79.7	79.1	78.8	78.0	79.4	85.1	78.2	80.0	78.5	79.4	6
USA/ArkDPI/1997	7	42.1	45.1	50.0	48.2	40.3	31.0		78.3	86.7	83.5	78.0	86.4	81.2	78.6	92.5	79.5	81.2	7
Netherland/D207/1989	8	48.2	56.2	63.3	56.2	52.2	28.6	48.6		79.5	78.0	79.5	78.6	82.7	82.1	79.2	79.5	82.4	8
USA/Gray/1983	9	49.1	55.7	58.7	55.3	50.0	23.2	58.0	56.6		84.7	79.2	99.1	80.9	80.4	88.1	82.4	81.2	9
USA/Holte	10	54.3	53.0	56.1	50.4	54.3	24.1	53.0	53.9	64.6		79.5	84.4	79.5	79.2	85.0	77.5	80.9	10
Italy/Italy-02/2002	11	57.5	66.9	50.4	69.6	52.2	23.4	44.2	50.8	53.0	55.7		78.6	76.6	88.1	78.9	79.2	82.4	11
USA/JMK/1993	12	49.1	55.7	59.6	56.2	50.0	23.2	58.0	53.9	97.3	64.6	52.2		81.2	79.8	87.8	82.4	80.6	12
USA/M41	13	58.0	55.7	99.1	54.8	62.8	32.1	50.0	63.3	58.7	56.1	50.4	59.6		77.5	82.4	80.6	80.4	13
UK/4/91/1998	14	61.9	67.8	51.3	65.7	53.9	22.6	46.4	58.5	58.9	54.8	74.1	58.0	51.3		79.5	81.2	82.1	14
USA/Cal99/2003	15	50.0	53.9	60.5	54.4	49.1	30.1	75.8	56.6	71.4	63.7	51.3	71.4	60.5	56.2		80.9	82.7	15
Brazil/IBV-USP-16/2007	16	56.6	60.7	54.8	57.6	53.9	24.3	48.2	58.0	63.3	51.3	53.5	63.3	54.8	60.3	59.8		79.8	16
China/QXIBV/1999	17	54.8	57.1	61.0	58.9	59.2	25.2	52.2	62.1	56.6	58.4	58.0	54.8	61.0	60.3	59.2	56.2		17
									Amino acid identity (%)										

* Highlighted in colors are the pairwise comparisons of nucleotide and amino acid identities of Cuban IBV isolates. The highest S1 sequence-based homologies identified among those isolates attending to these two parameters were: Cuba/La Habana/CB6/2009 to the recent North American genotype USA/DMV/5642/06 causing IBV respiratory disease in broilers; Cuba/La Habana/CB19/2009 to the Belgian nephropathogenic isolate Belgian/B1648/1996; and Cuba/La Habana/CB13/2009 to the North American IBV Mass genotype USA/M41.

neutralizing antibody expression [18] and haemagglutinating activity [19]. Its sequences are variable, and mutations in this region have been associated with changes in antigenicity and viral pathogenicity [20]. In Cuba, the presence of BCoV was first reported in 2006 [21], and sporadic outbreaks have continued to occur.

To study the genetic diversity and the phylogenetic relationships among different isolates of BCoV, a total of 30 samples collected between 2009 and 2011 (Figure 2) were used for amplification by PCR and direct sequencing of S gene partial or total. The comparison of sequences and phylogenetic studies were carried out with the employment of partial or complete sequences of S gene as phylogenetic markers. All Cuban BCoV strains were located in a same cluster, supported by 100 % bootstrap values and 1.00 posterior probability values. The Cuban BCoV strains were located in the same cluster as the USA BCoV strains EF424621 and EF424623, suggesting a common origin.

This phylogenetic cluster was also the only group of sequences in which no recombination events were detected. Of the 45 amino acid changes found in the Cuban strains, in comparison with the reference strain Mebus, four were unique.

Several of the changes found have been reported as markers of enteric tropism and respiratory, markers of virulence. From the unique changes found in the Cuban field strains, one of them was located in the heptad-repeat linked to higher efficiency in the viral replication as well as in a jump of host.

The fact that the rate of homologous recombination between coronavirus is very high [22] makes difficult the phylogenetic analysis, due to the problems of inference that carry coupled this type of elements (mosaics of recombination) for the relationships of kindred [23]. Nevertheless, the Cuban BCoV strains

are clustered with the USA BCoV strains, and this cluster was the only one to contain strains with a non-recombinant origin, indicating an epidemiological link between Cuban and USA BCoVs. Considering the insular condition of the country, the fact that BCoV is only transmitted by direct contact between animals and that the first evidence of BCoV infection in Cuba was obtained in 2004 [21] suggests that a USA BCoV strain(s) is the most likely origin of the Cuban BCoV strains.

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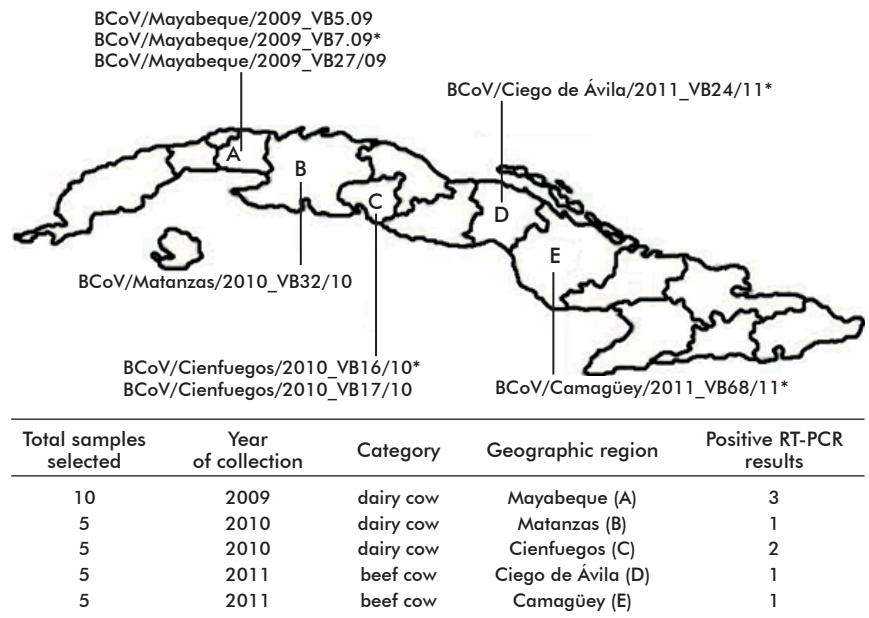| Total samples selected	Year of collection	Category	Geographic region	Positive RT-PCR results
10	2009	dairy cow	Mayabeque (A)	3
5	2010	dairy cow	Matanzas (B)	1
5	2010	dairy cow	Cienfuegos (C)	2
5	2011	beef cow	Ciego de Ávila (D)	1
5	2011	beef cow	Camagüey (E)	1

Figure 2. Cuban geographic distribution of the sample collection sites. The associated table indicates the quantity of samples analyzed from each area and the positive RT-PCR results obtained from each of them (*complete S gene sequenced samples).

Noteworthy, the group of sequences nearer to Cuban strains was isolated from wild ruminant in captivity [24, 25]. Therefore, the relationship between USA and the Cuban BCoV strains not only is restricted to the possible movement of cattle, but also results from possible movements of wild ruminant in captivity. However, the lack of additional information on movements of bovine and wild ruminant for captivity in zoological or natural parks, does not permit a categorical confirmation of this origin. Nevertheless, the certainty reached in the last years for the phylogenetic analysis [26] allows us to assume that the most probable origin for the introduction of the BCoV to Cuba was from strains of USA.

On the other hand, the molecular characterization carried out in the present work could explain the severity of the clinical signs that were presented during the outbreaks of the disease in the country. The findings of point mutations as 'molecular signatures' in the sequences of S protein of the Cuban strains offer relevant information not only to the national scientific community but to the coronovirology in the world, in the search of discoveries relating the gene sequences to viral phenotypes. Given the complexity of these agents, the fact of having a mechanism of replication by RNA nested subgenomics and a genomic RNA of great size suggests the necessity of analyzing and studying other genes of interest comparatively for a better understanding.

This study provides a good approach to the diversity of the BCoV strain circulating among Cuban herds. The putative origin of these strains and the molecular characteristics of the S protein supports the successful infections by this agent and the development of clinical disease that result in significant economic losses for the country.

Conclusions

The genetic diversity of IBV isolates is high given the low identity of sequences and the emergency of possible new genotypes in Cuba. The genetic diversity of the BCoV field strains studied in the different regions of Cuba is low based on their high sequence identity and their location in a unique cluster. The possible origin of the BCoV Cuban strains can be defined from USA strains that circulated during 2003 in wild ruminant, after their introduction in a simple independent event. Moreover, the presence of virulence markers and respiratory tropism in the Cuban sequences are in agreement with the clinical signs observed in the field.

Futures lines of development could be directed to perform a vaccination-challenge assay to know the efficacy of vaccine strain against the recent isolates Cuba/ La Habana/CB6/2009 and Cuba/La Habana/CB19/ 2009. Also to perform experimental studies to evaluate the pathogenicity of isolates Cuba/La Habana/ CB19/2009 and Cuba LaHabana/CB6/2009. The molecular characteristics and phylogenetic relationships in other genes (HE, M, E) of the BCoV strains and isolates studied need to be determined and their possible dual tropism elucidated. The prevalence of infection by these agents in the studied Cuban regions will be highly valuable.

Acknowledgments

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