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First evidence of the emergence of novel putative infectious bronchitis virus genotypes in Cuba

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ABSTRACT

The emergence of new infectious bronchitis virus (IBV) genotypes or serotypes along with the poor cross-protection observed among IBV serotypes have complicated the avian infectious bronchitis (IB) control programs in different geographic regions. In Cuba, the lack of genetic information regarding IBV and the increasing epidemiological importance of this virus in Cuban chicken flocks demand further characterization of IBV isolates. In the present work, studies of genetic diversity and phylogenetic relationships among recent IBV isolates from Cuban chicken flocks showing respiratory disorders were performed. 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.

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Avian infectious bronchitis (IB) is a highly contagious viral disease of poultry characterized by respiratory signs, nephritis and reduced egg production (Cavanagh and Naqi, 2003). This disease is caused by infectious bronchitis virus (IBV), a member of the genus *Gamma coronavirus*, family *Coronaviridae*, order *Nidovirales* (ICTV, 2009) which is considered a major pathogen in poultry production (OIE, 2008). The IBV genome consists of a single-stranded positive sense RNA molecule of approximately 27.6 Kb which encodes several nonstructural proteins involved in RNA transcription and replication and four structural proteins, spike (S), small membrane (E), membrane (M) and nucleoprotein (N) (Bourne et al., 1987). The S glycoprotein is formed by a globular S1 subunit that is anchored in the membrane by the S2 subunit (Cavanagh, 2007). The S1 subunit contains the main virus-neutralization epitopes, which are involved in the induction of neutralizing, serotype-specific haemagglutination inhibiting antibodies, and protective immunity (Cavanagh, 2003; Hodgson et al., 2004). Hence mutations within this genome region may result in the emergence of new viral variants (Moore et al., 1998).

The emergence of new IBV genotypes or serotypes (Lim et al., 2011; Mahmooda et al., 2011; Ducatez et al., 2009) along with the poor cross-protection observed among IBV serotypes (Cowen and Hitchner, 1975) have complicated the IB control programs

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(Cavanagh, 2007). In Cuba, the IB control strategy is based on the use of an attenuated live vaccine strain H120 of Massachusetts (Mass) serotype (reviewed in Colas et al., 2010) and despite of the extensive vaccination performed, the presence of IBV in vaccinated flock has been recently revealed by reverse transcriptase-polymerase chain reaction (RT-PCR) detection associated with clinical respiratory disorders in poultry (Acevedo et al., 2010). However, genetic information on IBV in Cuban chicken flocks is not still available.

The nucleotide sequencing followed by phylogenetic analysis based on the IBV S1 complete gene that contains the main virus-neutralization epitopes provide a fast and powerful method for the identification of genotypes and the prediction of serotypes.

Nucleotide sequencing of a diagnostically relevant fragment of the S1 gene is the most useful technique for the differentiation of IBV strains and has become the genotyping method of choice in many laboratories (OIE Manual, 2008). IBV phylogenetic clustering based on partial S1 gene sequences has been considered an accurate and less labor intensive method for the rapid identification of IBV field isolates and variants (Kulkarni and Resurreccion, 2010).

In the view of the lack of genetic information concerning IBV and the increasing epidemiological importance of this virus in Cuban chicken flocks further characterization of IBV isolates was considered necessary. Therefore, the objective of this study was to investigate the genetic diversity and the phylogenetic relationships among IBV isolates associated with respiratory disease from Cuban chicken flocks, based on partial S1 phylogenetic marker.

A total of 100 layers White Leghorn, between 140 and 475 days-old of four poultry flocks (25 layers/flock) were included in this study. The birds showed respiratory disorders and the flocks were under vaccination program based on the use of the IBV attenuated Mass serotype vaccine H120 (Labiofam, Cuba). The flocks were from La Habana which represents the major Cuban poultry region.

Swabs from the upper respiratory tract of 25 layers (five layers/swab pool) or tracheal and lung tissues from five birds were collected from each flock. The samples were prepared to be analyzed by a semi-nested RT-PCR (nRT-PCR) (Cavanagh et al., 2002) and virus isolation (VI) following standard procedures as described at the OIE Manual (OIE, 2008).

Total RNA was extracted from supernatants harvested from swabs, tissue samples, and allantoic fluids using TRI Reagent LS (SIGMA, San Louis, Missouri, USA), as recommended by the supplier. First strand complementary DNA (cDNA) was synthesized using Moloney-Murine leukemia virus reverse transcriptase (M-MLV RT) (Promega, Madison, WI, USA) according to manufacturer's instructions.

The presence of IBV in the samples collected was tested by semi-nested RT-PCR by using primers targeted to a region of the 39-untranslated region (UTR), highly conserved amongst IBV genotypes, as described by Cavanagh et al. (2002).

We processed upper respiratory tract swabs and tissue homogenates for VI in SPF embryonated chicken eggs, selected from the nRT-PCR positive samples, following the standards procedures described by the OIE Manual (OIE, 2008). The presence of IBV was determined by nRT-PCR.

Partial reverse transcription and amplification of the spike gene of IBV were carried out as described by Worthington et al. (2008), resulting in amplicons of 390 bp between nucleotides 705 and 1094 (amino acids 236–364) of the S1 coding region (for strain UK/7/93, GenBank accession Z83979). For this partial S1 RT-PCR, the IBV isolates obtained were selected.

Phylogenetic relationships among IBV genotypes were analyzed using Neighbor-Joining (NJ), Bayesian Inference (BI) and Maximum Likelihood (ML) methods. Additionally, the Cuban IBV nucleotide sequences were compared among them and with reference strains from different IBV genotypes using the BioEdit Sequence Alignment Editor (Hall, 1999).

The IBV genome was detected in three out of four flocks assessed by nRT-PCR. All tracheal swabs and tissues samples from the three detected flocks yielded positive results by the nRT-PCR assay. However, the virus was only successfully isolated from the

samples that yielded a positive amplification product after the first amplification round. Thus, three IBV isolates were obtained from three different flocks.

The nucleotide and amino acid identities of the Cuban IBV isolates obtained were 79.2–87% and 55.7–70.7%, respectively, when pairwise comparisons were performed among them (Table 1). These results suggest a high variability among viral strains circulating in the Cuban flocks assessed.

The partial S1 sequence of the isolate Cuba/La Habana/CB6/2009 exhibited the highest nucleotide (91.3%) and amino acid (78.3%) identities values with the new North American IBV genotype USA/DMV/5642/06 (Table 1) which emerged in the 2006 causing respiratory diseases in broilers from Delmarva, USA (Wood et al., 2009). On the other hand, the isolate Cuba/La Habana/CB19/2009 showed the highest nucleotide (87.8%) and amino acid (77.4%) identities with the Belgian nephropathogenic isolate B1648 (Table 1) which was associated with nephritis and a mortality rate in a range of 10–25% in H120-vaccinated and non-vaccinated flocks, respectively (Meulemans et al., 1987). Finally, the IBV isolate Cuba/La Habana/CB13/2009 exhibited the highest nucleotide (99.7%) and amino acid (99.1%) identities with the IBV Mass genotype.

The comparative analysis based on the deduced amino acid sequence of partial S1 sequence performed to assess the relation of the Cuba/La Habana/CB6/2009 and Cuba/La Habana/CB19/2009 isolates with the deduced amino acid sequence of Mass genotype showed that these Cuban isolates are 51% and 45%, respectively, different from the Mass genotype.

All three algorithms used for phylogenetic analysis showed the same topology from the S-H test, which was supported by moderate to high confidence values (Fig. 1). The isolates Cuba/LaHabana/CB6/2009, Cuba/LaHabana/CB13/2009 and Cuba/La Habana/CB19/2009 were grouped with the DMV5642 genotype, Mass genotype and B1648-like, respectively (Fig. 1). These results suggest that at least IBV strains similar to the DMV5642 genotype, Mass genotype and B1648-like are circulating in Cuban chicken flocks.

The emergence and spread of different IBV geno-serotypes have complicated the IB disease control. Several reports showed the continue emergence of new IBV variants caused by the evolution of the field strains (Dolz et al., 2008; Mahmooda et al., 2011) or by recombination events among heterologous strains classified into different genetic groups (Lim et al., 2011).

The presence of IB in Cuban chicken flocks was detected in the early eighties in intensive production systems associated to

Table 1
Nucleotide and deduced amino acid identities of IBV hypervariable region of the S1 gene.

	Percentage of nucleotides identity																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
1		87	81.8	80.6	91.3	83.7	76.6	78	76.9	80.1	80.1	76.6	82.1	81.2	78.6	80.6	79.2	1	Cuba/La Habana/CB6/2009
2	70.7		79.2	87.8	80.9	80	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/CB19/2009
3	58	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	Cuba/La Habana/CB13/2009
4	60.1	77.4	54.8		78.3	80	77.2	81.5	79.2	78.3	87.3	79.2	77.8	85.8	79.2	80.1	82.1	4	Belgian/B1648/1996
5	78.3	56.6	62.8	53.9		84.8	78.6	79.5	78.3	79.8	77.8	78	84.1	78.6	78.9	80.4	81.2	5	USA/DMV/5642/06
6	30.4	24.3	32.4	24.3	28.6		78.8	79.7	79.1	78.8	78	79.4	85.1	78.2	80	78.5	79.4	6	Japan/JP9758/2003
7	42.1	45.1	50	48.2	40.3	31		78.3	86.7	83.5	78	86.4	81.2	78.6	92.5	79.5	81.2	7	USA/ArkDPI/1997
8	48.2	56.2	63.3	56.2	52.2	28.6	48.6		79.5	78	79.5	78.6	82.7	82.1	79.2	79.5	82.4	8	Netherland/D207/1989
9	49.1	55.7	58.7	55.3	50	23.2	58	56.6		84.7	79.2	99.1	80.9	80.4	88.1	82.4	81.2	9	USA/Gray/1983
10	54.3	53	56.1	50.4	54.3	24.1	53	53.9	64.6		79.5	84.4	79.5	79.2	85	77.5	80.9	10	USA/Holte/
11	57.5	66.9	50.4	69.6	52.2	23.4	44.2	50.8	53	55.7		78.6	76.6	88.1	78.9	79.2	82.4	11	Italy/Italy-02/2002
12	49.1	55.7	59.6	56.2	50	23.2	58	53.9	97.3	64.6	52.2		81.2	79.8	87.8	82.4	80.6	12	USA/JMK/1993
13	58	55.7	99.1	54.8	62.8	32.1	50	63.3	58.7	56.1	50.4	59.6		77.5	82.4	80.6	80.4	13	USA/M41
14	61.9	67.8	51.3	65.7	53.9	22.6	46.4	58.5	58.9	54.8	74.1	58	51.3		79.5	81.2	82.1	14	UK/4/91/1998
15	50	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	USA/Cal99/2003
16	56.6	60.7	54.8	57.6	53.9	24.3	48.2	58	63.3	51.3	53.5	63.3	54.8	60.3	59.8		79.8	16	Brazil/IBV-USP-16/2007
17	54.8	57.1	61	58.9	59.2	25.2	52.2	62.1	56.6	58.4	58	54.8	61	60.3	59.2	56.2		17	China/QXIBV/1999
	Percentage of amino acid identity																		

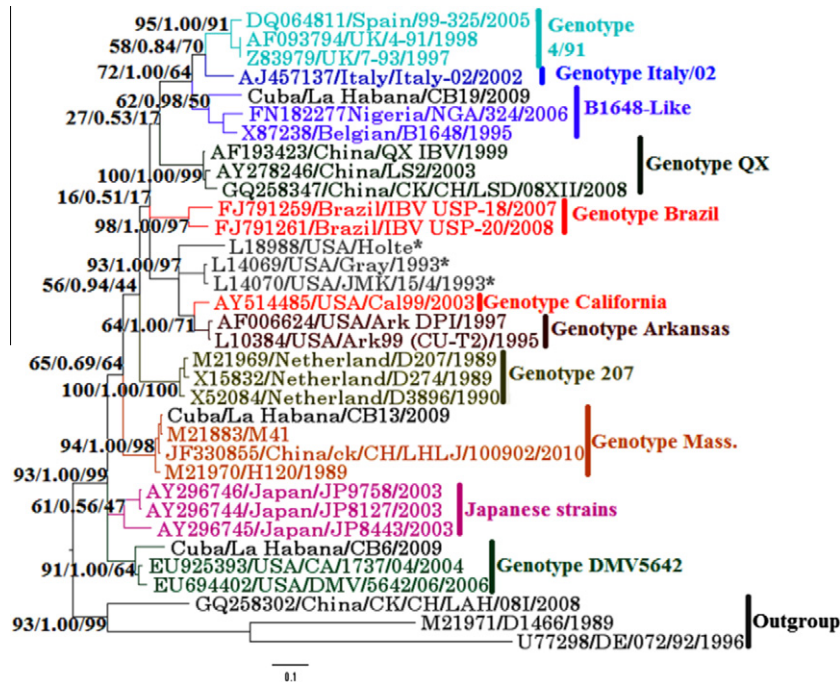


Fig. 1. Phylogenetic tree of IBV. IBV genotypes are indicated. The Cuban IBV sequences are denoted by black letter. A partial S1 sequence (390 bases) phylogenetic marker proposed by Worthington et al. (2008) was carried out. DNA sequencing reactions for both amplified strands were prepared using SX3+/SX4– primers and BigDye Terminator Cycle Sequencing Kit v.3.1, following the manufacturer's directions (Applied Biosystems). Sequencing products were read on an ABI PRISM-3100 Genetic Analyzer (Applied Biosystems). The Cuban IBV sequences were submitted to the EMBL/GenBank database under the Accession Numbers HE590762–HE590764 and were aligned together with sequence of the different IBV genotypes (accession number denoted) and references strain (*) published in the GenBank database, with the Clustal W program in the BioEdit Sequence Alignment Editor (Hall, 1999). ModelTest V.3.0.6 (Posada and Crandall, 1998) was used to estimate the best-fit model by the Akaike information criterion (AIC). Phylogenetic relationships among IBV genotypes were analyzed using Neighbor-Joining (NJ) and Bayesian Inference (BI) as described by Pérez et al. (2011). Additionally, Maximum Likelihood (ML) tree was computed using the PHYML v3.0 (Guindon and Gascuel, 2003), confidence was estimated by 1000 bootstrap replicates. The topologies were tested by the Kishino and Hasegawa test (K–H) (Kishino and Hasegawa, 1989) and the Shimodaira–Hasegawa test (S–H) (Shimodaira and Hasegawa, 1999), using the PAMLv4.3 program (Yang, 2007). 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.

chronic respiratory syndrome (Guilarte, 1985). The origin of IBV might be associated with the importation of different breeds and lines of chickens to be used for commercial chicken production. 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 (Guilarte, 1985). In the present work, the genetic analysis and the phylogenetic relationships among recent IBV Cuban isolates and reference IBV strains suggest that different variants of the virus are circulating in chicken flocks. Nevertheless, to determine if the Cuban isolates which grouped with the DMV5642 and B1648-like genotypes have emerged as consequence of evolution or recombination events require further studies.

Interesting results were the low degree of amino acid identities values found among the Mass genotype and two Cuban IBV recent isolates: Cuba/LaHabana/CB6/2009 and Cuba/La Habana/CB19/2009, indicative of a possible evasion to the host immune response due to a poor effectiveness of the vaccine based on the H120 strain, from the Mass genotype, applied in Cuban poultry flocks, against field isolates.

It has been reported that the S1 gene sequence comparison is a good predictor of cross-protection in chicken (Ladman et al., 2006). Nevertheless, Wood et al. (2009) have reported that a commercial live attenuated IBV vaccine containing the Massachusetts strain conferred protection against the challenge with the DMV/5642/06 strain, despite the fact that these two genotypes showed approximately 25% nucleotide divergence (Wood et al., 2009). Hence, it has been suggested that vaccination-challenge studies are the only way to evaluate the efficacy of a given vaccine strain for a field isolate (Wood et al., 2009). Therefore, to get insights in

the vaccine efficacy against the recent field isolates Cuba/LaHabana/CB6/2009 and Cuba/La Habana/CB19/2009 a vaccination-challenge trial will be required.

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. Nevertheless, experimental studies in order to assess the pathogenicity of the isolates Cuba/La Habana/CB19/2009 and Cuba/La Habana/CB6/2009 will be required with urgency.

In conclusion, the genetic diversity and phylogenetic relationships among IBV isolates which are circulating in Cuban chicken flocks associated with respiratory disorders were performed. Two putative genotypes genetically different to the Mass genotype were found in the flocks assessed. In addition, a potential nephropathogenic IBV isolate was found for first time in Cuba.

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