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Workshop: Infectious Bronchitis (IB) in the Brazilian Poultry Industry

Molecular Studies of the Brazilian Infectious Bronchitis Virus Isolates

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ABSTRACT

Avian infectious bronchitis virus (IBV) isolates have been widely characterized by reverse transcription followed by polymerase chain reaction and DNA sequencing. In present study, these techniques were applied to three viral genomic regions comprising the complete and/or a partial S1 segment, S2 and nucleocapsid genes. DNA sequences from viral isolates obtained from 1972 to 1989 and from 2006 to 2008 were compared. High similarity (>90%) was observed among some of the genomic segments, including S1 hypervariable region, which could suggest a common origin or ancestry. DNA sequences from S2 and N protein genes obtained from different infected tissues of the same flock were analyzed, and a clear segregation between respiratory and intestinal tract was observed. Therefore, these data suggest co-circulation of more than one viral strain in the same flock. 57.1% of DNA sequences from the S1 complete segment samples, 53.3% from the S2 fragment and 62.5% from the partial N gene were found to be different from analyzed sequences from reference strains leading to the conclusion that parte of viral isolates included in this study may be considered region specific. Considering the simultaneous analysis of the three genes, a large IBV genetic profile was observed in both old and recent isolates groups. However, most prominent diversity between viral isolates was obtained in the period from 1972 and 1989, showing the presence of a large number of variants in the state of Minas Gerais before the official approval of vaccination (1980).

INTRODUCTION

The infectious bronchitis virus (IBV) is a member of the *Coronaviridae* family, has a 27.6 Kb genome organized as a non-segmented, single-stranded and positive-sense RNA. The virion contains four structural proteins. The glycoprotein S consists of two subunits (S1 and S2) and presents a wide variety of antigenic determinants that may induce the production of specific neutralizing antibodies and also activate cell immunity (Holmes& Williams, 1990; Cavanagh, 1995; Ignjatovic & Sapats, 2005). However, most methodologies for serotype classification are based on the S1 subunit (De Wit, 2000). This protein structural conformation depends on its complement, the S2 subunit, which acts anchoring the S protein to the lipid envelope (Callisson *et al*., 1999; Cavanagh, 2007). In addition to S protein, nucleocapsid protein (N) is another important structural protein that may contribute to viral pathogenicity. The N protein is located inside the virion, closely associated with its genome, and interacts with viral and host-cell nuclear proteins (Holmes& Williams, 1990; Cavanagh, 1995; Ignjatovic & Sapats, 2005). Important biological functions are attributed to N-phosphoprotein, including viral replication, rRNA interaction, grouping and budding of

viral particles in the infected host cell. The N protein coding gene is highly conserved among different IBVs, which allows recurrent homolog recombination between different strains (Chen *et al*., 2005).

Despite the myriad of vaccination programs and control measures adopted, every year new infectious bronchitis outbreaks arise (Estevez *et al*., 2003; Gelb *et al*., 2005; Jackwood *et al*., 2005). Ideally, a vaccine election should be based on molecular and serological information collected from virus strains existing in the epidemic area. In present study, we characterized 28 IBV Brazilian isolates by means of viral genome sequencing (complete or part of the S1 gene and part of S2 and N segments), followed by DNA sequence alignment and dendrogram construction, including the IBV reference sequence available in the public database (GenBank, DDBJ).

MATERIAL AND METHODS

La Sota strain of Newcastle disease virus (NDV) and a pool of allantoic fluid (AF) of non-infected specific pathogen free (SPF) eggs were used as negative controls in all experiments. SPF eggs were kindly provided from Biovet S.A. Twenty eight field isolates of avian infectious bronchitis virus were isolated from clinical outbreaks in the Brazilian industrial poultry, from 1972 to 1989 and from 2006 to 2008. Bird tissue samples from the latter period were divided according to affected each organ (trachea, lung, kidney, cecal tonsils, intestine, and proventriculus) and tested as pools of 3-5 chickens derived from the same flock. All samples were obtained from chickens presenting gross lesions compatible with avian infectious bronchitis (IB). All experiments were performed using nine viral reference strains as positive controls: M41, Connecticutt-A5968 (Conn-A5968) and Arkansas-99 (Ark-99) from the American Type Culture Collection (ATCC); JMK, SE-17, Iowa-609 and Iowa-97 from SPAFAS (USA); H52 from United States Department of Agriculture (USDA/ USA); and 6-82 kindly provided by Dr. Jane A. K. Cook, of the Institute for Animal Health (Houghton Laboratory, UK). Viral production, RNA extraction, and RT-PCRs were performed according to Abreu (2006). The oligonucleotides used in PCR reactions were described by Zwaagstra *et al*. (1992), Kwon *et al*. (1993), and Lin *et al*. (1991), resulting in fragments sizes of 438 bp (N), 400 bp (S2), and 1720 bp (S1), respectively. Following PCR amplification, products were resolved in 1% agarose gel, and the specific bands were purified using Wizard® SV Gel and PCR Clean-Up System Kit (Promega Corporation, WI), according to manufacturer's protocol. After quantification of purified PCR products, DNA fragments were sequenced using ABI Prism® Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (PE Applied Biosystems Inc.). In order to prevent sequence errors due to polymerization and/or sequencing processes, each sample was amplified in two independent PCR reactions and the consensus sequence was deduced from, at least, three DNA reads. After analyses of the sequences quality using E-GENE and Phred, the resulting consensus sequences generated by Cap3 program were aligned using Muscle (Edgar, 2004).

In order to generate neighbor-joining dendrograms, the resulting alignments (19 S1 sequences, 21 S2 and14 N fragments) derived from the IBV nucleotide and amino acid sequences were used as inputs for Clustal X program (Jeanmougin *et al*., 1998). The GenBank accession numbers for partial nucleocapsid nucleotide sequences used in the comparison were: D1466, AF203006.1; D41, AY846837; Ark-99, M85244.1; ArkDPI, AY942745.1; Gray, S48137.1; Conn, AY942746.1; CU-T2, U04805.1; DE072, AF203001.1; Beaudette (M42), M28565; H52, AF352310.1; H120, AY028296.1; M41, AY851295.1; VicS, U52594.1; Q3/88, U52600.1. GenBank accession numbers for partial S2 spike glycoprotein nucleotide sequences used in the comparison were: Ark-99, AF094814.1.GI:4972604; Cav1013, AF239985; Conn46, AF094818; CU-T2, U04739; D207, X58003; D274, X15832; DE072, AF201930; GAV-92, AF094817; Gray, AF394180; Holte, AF334685; Iowa, AF334684; JMK, AF239982; SE17, AF239984; M41, AY851295; 6/82, X04723; H52, AF352315; KB8523, M21515; Turkey coronavirus strain G1, AY342357.1. DDBJ accession numbers for partial S2 spike glycoprotein nucleotide sequences used in the comparison were: Ark 4257, AF239983; Ark 3668, AF094816; Ark 3168, AF094815. GenBank accession numbers for complete and/or partial S1 spike glycoprotein nucleotide sequences used in the comparison were: Conn46, L18990; Ark-99, L10384; 4/91 pathogenic, AF093794; 4/91 attenuated, AF093793; CUT2, U04739; D41, AF036937; DE072, AF274435; GAV-92, U16157; Gray, L18989; Holte, L18988; JMK, L14070; Ma5, AY561713; M41; AY851295; H120, M21970.1; H52, AF352315; 6/82, X04723; Beaudette, DQ001334; Turkey coronavirus strain G1, AY342357.1; Partridge/GD/S14/2003; AY646283. The reliability of nodes in phylogenetic trees was evaluated using bootstrap values calculated from

10,000 resamplings of the alignment data. Nucleotide and amino acid identities were obtained using ClustalW (EMBL-EBI).

RESULTS AND DISCUSSION

In data analysis using the sequences of the 28 IBV isolates, the most informative dendrograms were those obtained using amino acid instead of nucleotide sequences. Considering all evaluated regions from the viral genome, older viral isolates (1972-1989) presented higher amino acid variation than the recently isolated samples (2006-2008). This may be explained by selective pressure derived from mass vaccination efforts. Nevertheless, high amino acid similarity (93% or higher) was found in all evaluated genomic regions in old isolates, obtained between 1975 and 1984, suggesting a putative common origin among those IBV strains; however, more data are required to confirm this hypothesis. In contrast, recent isolates presented low similarity with old IBV samples (1972 to 1989), showing a putative separate viral origin and co-circulation of many viruses in specific geographic regions. These results were also observed by other groups (Capua *et al*., 1999; Gelb *et al*., 2005).

In particular, dendrograms obtained from the N and S2 sequences were able to discriminate enteric from respiratory IBVs. Samples were extracted from different infected tissues of birds from a same flock, suggesting the presence of variant circulating strains. However, when a small portion of the S1 HVR (127 aa) from the same set of samples was analyzed, the observed difference among viruses from different tissues was not detected. This may be explained by the lack of information provided by the small portion of the S1 sequence, emphasizing the importance of analyzing large sequence fragments and the putative significance of S2 subunit in the viral tropism and pathogenicity. It was not possible to analyze viral tropism in old isolates (1972-1989) due to organs availability, since only samples of tracheas and lungs from chickens exhibiting respiratory distress and kidneys from birds presenting nephritis-nephrosis syndrome were available. Changes in IBV pathogenicity and tissue adaptation were previously reported in the literature, taking into account that genes N, S2, or M did not belong to the same serotype as the S1; however this matter is not fully understood (Yu *et al*., 2001a; b, Cavanagh, 2007; Mondal & Cardona, 2007).

In addition, the dendrogram based on the S1 segment showed a better resolution between isolates and reference strains, which was expected due to the higher variation of this segment as compared to the S2 and N genes (De Wit, 2000; Cavanagh, 2007). Regarding the S1 segment analysis, the following results were obtained: (1) 7 isolates were classified as Massachusetts strain and were kept in the same group, no matter using larger or smaller sequences (220 aa and 127 aa); (2) isolates 7 and 10 were considered very dissimilar from other reference strains, and were grouped in the same independent branch of the tree; (3) when 14 of the complete S1 segments (503 aa) were analyzed, only 4 strains were grouped with the Massachusetts reference strain; (4) 8 isolates were grouped in different genomic groups when dendrograms were built using smaller sequences as input (220 aa and 127 aa).

Considering all the three segments (S1, S2, and N) investigated in present study, 7 isolates seem to present putative gene recombination. However it is necessary to confirm the intergenic recombination event by sequencing larger gene segments, as extensively reported (Estevez *et al*., 2003; Huang *et al*., 2004; Shieh *et al*., 2004; Jackwood *et al*., 2005).

Moreover, dendrogram analysis confirmed the existence of regional Brazilian IBV isolates that are distinct from isolates previously described around the world. This finding is consistent with those of Jackwood *et al*. (2005), who identified new IBV strains in North America.

CONCLUSIONS

In summary, this study consisted of sequencing analysis of three different genomic regions of 28 Brazilian IBV isolates from old and recent outbreaks (1972 to 1989 or 2006 to 2008). It was verified that 6, 16, and 8 isolates were grouped with reference strains including vaccine strains, when the complete sequence of S1, partial S2, and N were considered, respectively. In addition, three groups, of 8, 10, and 7 isolates, correlated with specific outbreaks and geographic regions. Furthermore, these findings are time-independent. Wide genetic diversity was observed when isolates and reference strains were compared. In addition, some of the analyzed samples possessed genome segments that were specific of more than one serotype, suggesting possible recombination events and distinct origin. Some recent isolates presented high similarity (> 93%) with old isolates (1975 to 1984), indicating putative common ancestry or origin; however, this hypothesis requires further investigation.

In the present study, it was aimed to show that the use of larger S1 sequences is essential for IBV strain classification in contrast with the misguided use of small fragments; furthermore, the analysis of more than one gene is advisable for genomic classification.

Interestingly, different tissues from a same fowl presented different S2 and N sequences, with respiratory and digestive tract IBV sequences showing evident separation, suggesting viral tissue tropism. This result was not verified in the S1 sequence analysis.

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