Molecular Characterization of Infectious Bronchitis Virus Strains Isolated from the Enteric Contents of Brazilian Laying Hens and Broilers

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Molecular Characterization of Infectious Bronchitis Virus Strains Isolated from the Enteric Contents of Brazilian Laying Hens and Broilers


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SUMMARY. Infectious bronchitis virus (IBV) is the causative agent of avian infectious bronchitis, which is characterized by respiratory, reproductive, and renal signs. However, the role of IBV as an enteric pathogen is still controversial. In Brazil, antigenic groups of IBV divergent from the Massachusetts serotype used for vaccination schedules in that country have already been demonstrated. The present study aimed to assess the different genotypes of IBV in Brazilian commercial poultry flocks by partial sequencing of the S1 amino-terminus coding region using enteric contents as samples and examine their relationship with the vaccine serotype currently in use. Samples of enteric contents were taken as pools of five birds from each of 18 poultry farms (17 broiler and one laying farm) from five Brazilian states between 2002 and 2006. Birds were presenting watery diarrhea and poor general condition but were without respiratory, renal, or reproductive signs. Conventional antibacterial and anticoccidial therapies were unsuccessful and, furthermore, all samples proved negative for rotavirus, reovirus, and astrovirus. Eleven IBV samples were isolated in embryonated eggs and resulted in S1 sequences. Phylogenetic analysis showed that these segregated into an exclusive cluster, close to serotype D274, but distant from Massachusetts. Mean amino acid identity amongst these Brazilian strains was 94.07%; amongst these and serotypes D274, 4/91, and Massachusetts, mean amino acid identity was 77.17%, 69.94%, and 68.93%, respectively. In conclusion, the presence of genotype variant strains of IBV in Brazilian poultry flocks has been demonstrated and might be the reason for the unsuccessful control of IBV in Brazil. Furthermore, these results also strengthen the implications of IBV in enteric diseases of poultry.

RESUMEN. Nota de Investigación—Caracterización molecular de cepas del virus de bronquitis infecciosa aislados de contenidos intestinales de ponedoras y pollos de engorde en Brasil.

El virus de bronquitis infecciosa es el agente causal de la bronquitis infecciosa aviar, que se caracteriza por la presencia de signos respiratorios, reproductivos y renales. Sin embargo, el papel del virus de bronquitis infecciosa como patógeno entérico sigue siendo controvertido. En Brasil ya se han demostrado grupos antígenicos del virus de bronquitis infecciosa diferentes al serotipo Massachusetts utilizado en los planes de vacunación. El objetivo del presente estudio fue identificar los diferentes genotipos del virus de bronquitis infecciosa presentes en las parvadas comerciales de Brasil, mediante secuenciación parcial del gen S1 utilizando muestras de contenidos intestinales y examinando su relación con los serotipos vacunales utilizados actualmente. Entre los años 2002 y el 2006 se tomaron muestras de contenido entérico de grupos de cinco aves provenientes de 18 granjas avícolas (17 de pollos de engorde y una de ponedoras) pertenecientes a cinco estados de Brasil. Las aves presentaban diarrea acusa y una mala condición general, pero no mostraban signos respiratorios, reproductivos o renales. Las terapias antibacteriales y anticoccidiales convencionales fallaron y además, todas las muestras resultaron negativas a rotavirus, reovirus y astrovirus. Once virus de bronquitis infecciosa fueron aislados en huevos embrionados de los cuales se obtuvo la secuencia del gen S1. Los análisis filogenéticos mostraron que todos los aislamientos se ubicaron en un grupo exclusivo, cercano al serotipo D274, pero distante del serotipo Massachusetts. El promedio de identidad de aminoácidos entre las cepas de Brasil fue de 94.07%. Entre estas cepas y los serotipos D274, 4/91 y Massachussets, el promedio de identidad de aminoácidos fue de 77.17%, 69.4% y 68.93%, respectivamente. En conclusión, se demostró la presencia de genotipos variantes de cepas del virus de bronquitis infecciosa en parvadas de aves domésticas en Brasil, pudiendo ser esta la razón de las fallas en el control del virus de bronquitis infecciosa en el país. Además, estos resultados refuerzan las implicaciones del virus de bronquitis infecciosa en enfermedades entéricas de las aves.

Key words: avian infectious bronchitis virus, molecular characterization, enteritis

Abbreviations: IB = infectious bronchitis; IBV = infectious bronchitis virus; RT-PCR = reverse transcription–polymerase chain reaction; S = spike; USP = University of São Paulo; UTR = untranslated region

Infectious bronchitis virus (IBV), the causative agent of avian infectious bronchitis (IB), has more than 20 different serotypes that emerged from different events of insertions, deletions, substitutions, and RNA recombinations and which have been recognized worldwide (1,14,28). This large diversity of serotypes leads to vaccine failures responsible for new outbreaks and to the difficulties in controlling the disease (7). Avian IB affects mainly the respiratory, reproductive, and, sometimes, renal systems of broilers, layer, and breeder chickens, causing severe economic losses to the poultry industry, but the implications of IBV infection in the enteric tract are still controversial (4,7).
IBV is a group 3 coronavirus with a single-stranded positive-sense RNA with 27 kb, which codes for nonstructural proteins involved in RNA transcription and replication and three envelope proteins, of which the spike (S) protein is the most antigenically and immunologically relevant one (27,30). In Brazil, the only vaccine strain approved by the Agriculture Ministry belongs to the Massachusetts serotype. Even so, not only has the existence of antigenic groups divergent from Massachusetts serotype already been demonstrated in this country (11), but molecular studies have shown that point mutations and insertion–deletion events in the hypervariable regions have led to the emergence of different genotypes (31), highly divergent from Massachusetts serotype (34).

The aim of the present study was to assess the different genotypes of IBV in Brazilian commercial poultry flocks using enteric contents as samples based on partial sequencing of the S1 amino-terminal coding region and their relationship with the vaccine serotype currently in use in this country.

### MATERIALS AND METHODS

**Case history and samples.** Between 2002 and 2006, 18 poultry farms (17 broiler and one laying farm) from three Brazilian states (Table 1) reported outbreaks of moderate-to-severe enteric disease. Birds showed watery diarrhea, poor general condition, broken and ruffled feathers, growth retardation, and increased feed conversion rate. No respiratory or reproductive signs were evident. No improvement was achieved after antibacterial or anticoagulants treatments.

Samples of enteric contents (pool 1) and respiratory tissues and kidneys (pool 2) were taken as pools of five birds after necropsy from each of the 18 farms and sent to the Laboratory of Avian Pathology of the University of São Paulo (USP) under refrigeration. Pools of enteric contents were also collected from 10 healthy flocks, in which none of the signs described for the ill flocks were found. The samples were prepared as 20% w/v suspensions in phosphate-buffered saline (0.01 M, pH 7.4) and clarified at 1,500 × g for 15 min; the supernatants were then collected for analysis.

Birds from farms 6 and 9 (Table 1) were vaccinated against IB with the Massachusetts serotype at day 1; in the other farms, no IBV vaccine was used.

**Reference strains.** Virus reference strains used as positive control included the Massachusetts vaccine strain of IBV, Nebraska calf diarrhea virus rotavirus strain, strain S1135 of revovirus, and a field strain of turkey astrovirus (33).

**RNA extraction.** Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions, directly from the supernatant of the samples suspensions, from the allantoic fluid of embryonated eggs for viral isolation from negative control (ultrapure water), and from reference strains.

**Virus screening.** The presence of IBV was screened by a reverse transcription–polymerase chain reaction (RT-PCR) aimed at the amplification of 179 bp of the 3′ untranslated region (UTR) using primers UTR 41+, UTR 31 −, and UTR 31+ and reaction conditions described by Cavanagh et al. (6). Avian astrovirus was surveyed using an RT-PCR targeted to the RdRp gene as described by Koci et al. (23).

In order to avoid any laboratory contamination, each step (RNA extraction, reverse transcription, and PCR, nested and electrophoresis) was carried out in separate rooms with separate materials.

The products of nested PCR were resolved in 1.5% agarose gel stained with 0.5 μg/ml ethidium bromide. Polyacrylamide gel electrophoresis was used to detect rotavirus and reovirus segmented RNAs according to the protocol described by Hertz et al. (17).

**Viruses isolation.** Briefly, the supernatants of IBV-positive samples (as determined by RT-PCR) were filtered through 0.45-μm and 0.22-μm membranes; 10 μg/μl of gentamicin was added, and 0.2 ml was inoculated into 10-day-old specific-pathogen-free embryonated chicken eggs (Biovet™ Laboratory, Vargem Grande Paulista, Brazil) by the allantoic cavity route. Eggs were incubated at 37°C and candled daily to check for embryo viability. Allantoic fluids were harvested 48–72 hr postinoculation and two further blind serial passages were performed in a similar way.

Each passage (allantoic fluid) was tested for the presence of IBV by the RT-PCR described above.

**PCR to the IBV S1 coding region.** The third passage of each sample that resulted positive for the presence of IBV in the allantoic fluid after RT-PCR to the 3′ UTR was submitted to another RT-PCR for the amplification of a segment of 706 bp of the S1 subunit coding region (nucleotides 25 to 730) using a combination of primer sense S1OLIGO 5′ (25) and anti-sense CK2 (22) and reaction conditions described by Ziegler et al. (37).

**DNA sequencing and phylogenetic analysis.** The PCR products corresponding to the S1 region were purified with the QiAquick Spin Miniprep kit (Qiagen, Valencia, CA) and submitted to bidirectional DNA sequencing with DYEnamic ET Dye Terminator (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer’s instructions; the sequences were resolved in a MegaBACE™ 1000 (Amersham Biosciences).

Aminoacidic neighbor-joining distance tree with a Kimura 2-parameter model was built with Mega 2.1 (24) with 1000 bootstrap replicates with the sequences of S1 and 29 homologous sequences retrieved from GenBank aligned with the Clustal/W method with Bioedit (16).

**Reference S1 nucleotide sequence from the GenBank included:**
- AF006624.1, AY851295.1, L14070.1, AF027512, AF274435.1, AF383717, AF383718, L18990.1, U04739.1, AY561713.1, AY514485.1, M21970.1, DQ001338.1, DQ064810.1, AY619894.1, AF093794.1, AF093793.1, X15832.1, DQ064813.1, AY427818.1, AF352315.1, AY352315.1, AY397529.1, AF286303.1, DQ070839.1, AF193423.1, AY427818.1, AY257061.1, AY250005.1, AY606322.1, and DQ001334.1.
RESULTS

Virus screening. All 18 samples of enteric contents (pool 1) were positive for IBV by 3’ UTR PCR and negative for rotavirus, reovirus, and astrovirus. The samples of respiratory tissues (lungs and tracheas) and kidneys (pool 1) were all negative for IBV. Furthermore, the samples of the 10 healthy flocks were all negative for the viruses surveyed in this study.

Virus isolation. Virus isolation in chicken embryos for all 18 enteric samples produced lesions characteristic of IBV, such as curled, hemorrhagic, and stunted embryos, usually by the third day post-inoculation of the third passages. IBV isolation was confirmed by the 3’ UTR PCR of the allantoic fluids of these passages in all 18 samples.

Phylogenetic analysis. Eleven of the 18 IBV samples in allantoic fluids resulted in a fragment of 706 bp of the S1 coding region, whereas the remaining seven samples produced no bands after this RT-PCR. The sequences obtained were assigned GenBank accession numbers DQ492307, DQ492308, DQ492309, DQ492310, DQ492311, DQ492312, DQ492314, and DQ492316 and the respective strains were named IBV/Brazil/2005/USP-02 to IBV/Brazil/2005/USP-18 (Table 1).

In the amino acid tree shown in Fig. 1, these 11 Brazilian strains segregated in an exclusive cluster, supported by a bootstrap value of 97, close to serotype D274, but distant from the Massachusetts and 4/91 serotypes clusters and from the other serotypes isolated worldwide.
Mean amino acid identity amongst the Brazilian strains was 94.07% and amongst these and serotypes D274, 4/91, and Massachusetts it was 77.17%, 69.94%, and 68.93%, respectively.

**DISCUSSION**

In spite of the intensive vaccination programs with vaccines of the Massachusetts type—the only one authorized by the Brazilian government—there are frequent outbreaks of the disease attributed to IBV in the field.

The RT-PCR results showed the presence of IBV in the intestinal contents of all surveyed birds in which classical respiratory, reproductive, and renal signs of IB were absent, but diarrhea was present. It is also noteworthy that no IBV was detected in samples of respiratory tract or kidneys. Taking into account that all samples were negative for reovirus, rotavirus, and astrovirus, it can be suggested that IBV was the only viral pathogen present in the samples. Furthermore, as antimicrobial and anticoagulant therapies were unsuccessful, enteropathogenic bacteria and coccidia can be ruled out as a cause for the signs observed.

Although it is known that IBV can replicate in the intestinal tract of chicken, this virus has not been associated with enteric disease. For instance, according to Ambali and Jones (2), a specific IBV strain (strain G) is able to replicate in all regions of the gut; on the other hand, another IBV isolate has been associated with proventricularis (4,37).

The results of virus isolation in chicken embryos show that the viruses were viable and infectious. Nonetheless, because enteric tissue samples were not taken from the birds surveyed in the present study for histopathological or immunohistochemical analysis, the replication of these IBV strains in enterocytes still needs confirmation.

Interestingly, all 11 Brazilian strains included in the phylogenetic analysis were found distant from the Massachusetts serotype, as shown in the phylogenetic tree (Fig. 1); accordingly, the amino acid identity between these two groups was as low as 68.93%.

The finding of an IBV genotype different from Massachusetts in the vaccinated birds (farms 6 and 9) and, importantly, in the unvaccinated birds, discards the possibility of detection of the virus contained in the vaccine instead of field strains of IBV. A direct consequence of this low amino acid identity is likely to be the failure of vaccination with a strain from the Massachusetts serotype to protect. Once the S1 subunit region targeted by DNA sequencing and its putative amino acid sequence harbor domains responsible for receptor binding, serotype definition, and virus neutralization (3,5,19,20,35), such a region can be used as a prediction for the receptor binding, serotype definition, and virus neutralization (3,5,19,20,35), such a region can be used as a prediction for the efficacies of vaccines because the degree of cross-protection declines (3,5,19,20,35).

Despite that, a question that remains to be answered is whether the IBV strains detected in the present study were the cause of the enteric disease reported. If so, does this represent an emerging and important pathogenic pattern of IBV?

The evolutionary trend from respiratory-to-enteric and vice-versa has already been reported for other coronaviruses, such as bovine coronavirus (8,15,29), transmissible gastroenteritis virus (32,36), and human coronavirus OC43 (9).

Of closer relevance, Australian IBVs changed the pathogenic type over 20 yr from nephropathogenic to respiratory by a subtle gene change (18). Other avian coronaviruses closely related to IBV, such as pheasant coronavirus and turkey coronavirus, also harbor enteric tropism and pathogenicity (6).

Taking into account these examples of evolution of certain coronaviruses, the fact that in the surveyed samples no other enteric virus was found, that bacteria and coccidia could be disregarded as etiologic agents, and that no IBV was detected in the enteric samples from flocks without enteric disease, one can speculate that the IBV strains detected here are likely to have played a role or even caused the enteric disease. However, further work needs to be conducted in order to test this hypothesis.

The Brazilian strains reported here appear to be more closely related to the D274 serotype, a European virus that is unrelated to the Massachusetts serotype. It therefore becomes clear that the vaccination programs used in Brazil for control of IB must be updated with serotypes more appropriate for the important indigenous Brazilian types and different from the conventional Massachusetts vaccine. An alternative could be the use of a vaccination schedule based on two different serotypes producing a broad spectrum of protection (10,11,21).

In conclusion, the presence of genotype variant strains of IBV in Brazilian poultry flocks has been demonstrated and might be the reason for the frequent outbreaks of IB in Brazil because of ineffective control methods based on monovalent vaccination with the Massachusetts serotype.

**REFERENCES**


