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Emergence of a New Genotype of Avian Infectious Bronchitis Virus in Brazil

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SUMMARY. Infectious bronchitis virus (IBV) is the agent of a highly contagious disease that affects domestic fowl (*Gallus gallus*). Recent reports showed a high prevalence of one main IBV genotype (Brazil or BR-I) with low genetic diversity in commercial poultry flocks from Brazil. This research analyzed IBV positive poultry flocks from different rearing regions to verify the S1 gene variability and geographic distribution of variant IBV strains in recent years (2010 and 2011). Samples of IBV-positive flocks were obtained from 60 different farms. Forty-nine partial S1 gene sequences were determined and aligned for phylogenetic and amino acid similarity analyses. Eleven samples (22.4%) were similar to Massachusetts vaccine strains (Mass genotype) and 34 samples (69.4%) to the previously characterized Brazilian BR-I genotype. Interestingly, the remaining four samples (8.2%) clustered into a new IBV variant genotype (Brazil-II or BR-II), divergent from the BR-I. A unique nucleotide sequence insertion coding for five amino acid residues was observed in all the Brazilian variant viruses (BR-I and BR-II genotypes). These results show a higher genetic diversity in Brazilian IBV variants than previously described.

RESUMEN. Aparición de un nuevo genotipo del virus de la bronquitis infecciosa aviar en Brasil.

El virus de la bronquitis infecciosa (IBV) es el agente de una enfermedad altamente contagiosa que afecta a las gallinas domésticas (*Gallus gallus*). Los reportes recientes muestran una alta prevalencia de un genotipo principal de este virus (Brasil o BR-I) con baja diversidad genética en las parvadas avícolas comerciales de Brasil. Esta investigación analiza las parvadas avícolas positivas a la presencia de este virus en diferentes regiones, para verificar la variabilidad del gene S1 y la distribución geográfica de las cepas variantes de este virus en los últimos años (2010 y 2011). Se recolectaron muestras del virus de bronquitis de parvadas positivas en 60 granjas diferentes. Se determinaron 49 secuencias parciales del gene S1 y se alinearon para llevar a cabo análisis filogenéticos y de similitud de aminoácidos. Once muestras (22.4%) fueron similares a la cepa vacunal Massachusetts (Mass) (genotipo Mass) y 34 muestras (69.4%) fueron similares a la cepa brasileña previamente caracterizada, genotipo BR-I. De manera interesante, las cuatro muestras restantes (8.2%) se agruparon en un nuevo genotipo variante (Brasil-II o BR-II), que es divergente de la BR-I. Una inserción única en la secuencia de nucleótidos que codifica para cinco residuos de aminoácidos se observó en todos los virus variantes de Brasil (genotipos BR-I y BR-II). Estos resultados muestran una mayor diversidad genética de las cepas variantes brasileñas del virus de la bronquitis infecciosa a diferencia de lo que se había descrito anteriormente.

Key words: infectious bronchitis virus, genotype, variant, chicken, characterization

Abbreviations: Conn = Connecticut; E = envelope; GuSCN = guanidine isothiocyanate; HVR1/2 = hypervariable region 1/2; IB = infectious bronchitis; IBV = infectious bronchitis virus; M = membrane; Mass = Massachusetts; N = nucleocapsid; nt = nucleotides; RT-PCR = reverse transcription-PCR; S = spike

Infectious bronchitis (IB) is an acute and highly contagious viral disease that affects domestic fowl (*Gallus gallus*) worldwide. IB virus (IBV) initially infects the upper respiratory tract, but lesions can also affect the enteric and urinary tracts (5). The clinical signs of the IB include cough, nasal discharge, watery eyes, reduced egg quality, decreased feed conversion efficiency, and increased carcass condemnations that reduce the performance of the flocks and determine the economic impact of this disease in the poultry industry (11).

IBV is a *Coronavirus* (family *Coronaviridae*, and order *Nidovirales*) and has a single stranded RNA genome with positive sense and about 27.6 Kb in length that encode four structural proteins: nucleocapsid (N), membrane (M), envelope (E), and spike (S). The N phosphoprotein is encoded by a highly conserved gene and plays an important role in viral replication. The M protein participates in the assembly of infectious particles. The E protein, like the M protein, is important for the production of new infectious particles. The S protein is cleaved into two subunits: S1 and S2 with approximately 535 and 625 amino acids, respectively. The S1 glycoprotein is important in adsorption to the cellular receptor and virus entry into the host cell, besides inducing neutralizing antibodies. S1 is encoded by a highly variable gene among the different viral strains and is directly related to the diversity of IBV serotypes (5).

Genetic diversity of this virus, mainly in the S1 gene, was demonstrated in different poultry-producing regions of the world. Historically, serotypes Massachusetts (Mass) and Connecticut (Conn) were the first isolates in the 1940s and 1950s, respectively. In the following decades, several new IBV variants were identified and associated with the disease (8,9). In Brazil, IBV from the serotype Mass was originally identified in 1957 (17). Since then, different serotypes and viral variants were identified in all poultry-producing regions of the country as well as in some neighbor countries (2,12,16,18). Two recent studies demonstrated that the great majority of the IBV strains from commercial flocks with IB clinical signs in different states from Brazil in a period of 7 yr (2003 to 2009) were variants with a high similarity in the S1 glycoprotein gene, suggesting the prevalence of a unique genotype called Brazil (19) or BR-I (7).

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Table 1. Information from Brazilian IBV field samples analyzed in the present study.

Identification	Year of detection	Sample tissue	State[A]	Region[B]	RT-PCR S1	Group	GenBank Accession No.
IBV/BRAZIL/2010/SB-1558	2010	Kidney and trachea	SC	S	Positive	BR-I	JX559784
IBV/BRAZIL/2010/SB-1875	2010	Trachea	AL	NE	Positive	Mass	JX559785
IBV/BRAZIL/2010/SB-1888	2010	Trachea	RS	S	Negative	—	—
IBV/BRAZIL/2010/SB-1974	2010	Trachea	BA	NE	Positive	BR-I	JX559786
IBV/BRAZIL/2010/SB-1994	2010	Trachea	AL	NE	Positive	BR-I	JX559787
IBV/BRAZIL/2010/SB-2017	2010	Organs pool	RS	S	Positive	BR-I	JX559788
IBV/BRAZIL/2010/SB-2898	2010	Kidney and cecal tonsils	PR	S	Positive	BR-I	JX559789
IBV/BRAZIL/2010/SB-2925	2010	Kidney and trachea	SC	S	Positive	Mass	JX559790
IBV/BRAZIL/2010/SB-3730	2010	Trachea and cecal tonsils	SP	SE	Positive	BR-I	JX559791
IBV/BRAZIL/2010/SB-3735	2010	Trachea and swab of the trachea	RS	S	Negative	—	—
IBV/BRAZIL/2010/SB-4570	2010	Lung and trachea	SP	SE	Positive	BR-I	JX559792
IBV/BRAZIL/2010/SB-5258	2010	Trachea, lung, and kidney	SP	SE	Positive	—	—
IBV/BRAZIL/2010/SB-5261	2010	Trachea	RS	S	Positive	BR-I	JX559830
IBV/BRAZIL/2010/SB-5264	2010	Trachea	RS	S	Positive	BR-I	JX559793
IBV/BRAZIL/2010/SB-5267	2010	Trachea	RS	S	Negative	—	—
IBV/BRAZIL/2010/SB-5273	2010	Trachea	RS	S	Negative	—	—
IBV/BRAZIL/2010/SB-5291	2010	Trachea	RS	S	Negative	—	—
IBV/BRAZIL/2010/SB-5292	2010	Trachea	RS	S	Negative	—	—
IBV/BRAZIL/2010/SB-5293	2010	Trachea	RS	S	Positive	BR-I	JX559794
IBV/BRAZIL/2010/SB-5294	2010	Trachea	RS	S	Positive	BR-I	JX559795
IBV/BRAZIL/2010/SB-5335	2010	Trachea and kidney	SC	S	Negative	—	—
IBV/BRAZIL/2010/SB-5340	2010	Trachea, lung, and kidney	MG	SE	Negative	—	—
IBV/BRAZIL/2010/SB-5528	2010	Trachea	RS	S	Positive	BR-I	JX559796
IBV/BRAZIL/2010/SB-5530	2010	Trachea	RS	S	Positive	BR-I	JX559797
IBV/BRAZIL/2010/SB-5588	2010	Trachea	RS	S	Positive	—	—
IBV/BRAZIL/2010/SB-5677	2010	Kidney and trachea	SC	S	Negative	—	—
IBV/BRAZIL/2010/SB-5679	2010	Trachea, lung, and kidney	RS	S	Positive	BR-I	JX559831
IBV/BRAZIL/2010/SB-5808	2010	Kidney and trachea	SC	S	Positive	BR-I	JX559832
IBV/BRAZIL/2011/SB-A0297	2011	Trachea, cecal tonsil, lung	PR	S	Positive	Mass	JX559798
IBV/BRAZIL/2011/SB-A0395	2011	Cecal tonsil	PR	S	Positive	Mass	JX559799
IBV/BRAZIL/2010/SB-A0399	2010	Lung, trachea, and reproductive tract	SC	S	Positive	BR-I	JX559800
IBV/BRAZIL/2010/SB-A0451	2010	Trachea, cecal tonsil	BA	NE	Positive	BR-I	JX559801
IBV/BRAZIL/2011/SB-A0458	2011	Cecal tonsil	RS	S	Positive	BR-I	JX559802
IBV/BRAZIL/2011/SB-A0473	2011	Cecal tonsil	RS	S	Positive	BR-I	JX559803
IBV/BRAZIL/2010/SB-A0498	2010	Cecal tonsil	GO	CW	Positive	BR-I	JX559804
IBV/BRAZIL/2010/SB-A0552	2010	Trachea	GO	CW	Positive	BR-I	JX559805
IBV/BRAZIL/2011/SB-A0571	2011	Lung	PR	S	Positive	BR-I	JX559806
IBV/BRAZIL/2010/SB-A0588	2010	Trachea, cecal tonsil	BA	NE	Positive	BR-I	JX559807
IBV/BRAZIL/2010/SB-A0680	2010	Cecal tonsil	GO	CW	Positive	BR-I	JX559808
IBV/BRAZIL/2011/SB-A0797	2011	Lung, trachea, and reproductive tract	SC	S	Positive	BR-I	JX559809
IBV/BRAZIL/2010/SB-A1140	2010	Cecal tonsil	PR	S	Positive	BR-I	JX559810
IBV/BRAZIL/2010/SB-A1971	2010	Trachea, cecal tonsil, lung	BA	NE	Positive	BR-I	JX559811
IBV/BRAZIL/2010/SB-A2150	2010	Cecal tonsil	PR	S	Positive	BR-I	JX559812
IBV/BRAZIL/2010/SB-A2160	2010	Trachea, lung	PR	S	Positive	BR-I	JX559813
IBV/BRAZIL/2010/SB-A2240	2010	Trachea, lung	PR	S	Positive	BR-I	JX559814
IBV/BRAZIL/2010/SB-A2308	2010	Cecal tonsil	MT	CW	Positive	Mass	JX559815
IBV/BRAZIL/2010/SB-A2360	2010	Cecal tonsil	PR	S	Positive	BR-I	JX559816
IBV/BRAZIL/2010/SB-A2398	2010	Cecal tonsil	MT	CW	Positive	BR-II	JX559817
IBV/BRAZIL/2010/SB-A2400	2010	Cecal tonsil	PR	S	Positive	BR-I	JX559818
IBV/BRAZIL/2010/SB-A2401	2010	Cecal tonsil	MT	CW	Positive	BR-II	JX559819
IBV/BRAZIL/2010/SB-A2479	2010	Cecal tonsil	PR	S	Positive	BR-I	JX559820
IBV/BRAZIL/2010/SB-A2960	2010	Cecal tonsil	MT	CW	Positive	BR-II	JX559821
IBV/BRAZIL/2010/SB-A2962	2010	Cecal tonsil	MT	CW	Positive	BR-II	JX559822
IBV/BRAZIL/2010/SB-A3507	2010	Cecal tonsil	PR	S	Positive	BR-I	JX559823
IBV/BRAZIL/2010/SB-A3513	2010	Cecal tonsil	PR	S	Positive	Mass	JX559824
IBV/BRAZIL/2011/SB-A4016	2011	Trachea	GO	CW	Positive	Mass	JX559825
IBV/BRAZIL/2011/SB-A4069	2011	Trachea	GO	CW	Positive	Mass	JX559826
IBV/BRAZIL/2011/SB-A4072	2011	Trachea	GO	CW	Positive	Mass	JX559827
IBV/BRAZIL/2011/SB-A5004	2011	Kidney	BA	NE	Positive	Mass	JX559828
IBV/BRAZIL/2010/SB-A6540	2010	Trachea swab	SC	S	Positive	Mass	JX559829

[A]Brazilian states: SC = Santa Catarina; AL = Alagoas; RS = Rio Grande do Sul; BA = Bahia; PR = Paraná; SP = São Paulo; MG = Minas Gerais; GO = Goiás; MT = Mato Grosso.

[B]Brazilian regions: S = south; NE = northeast; SE = southeast; CW = center-west.

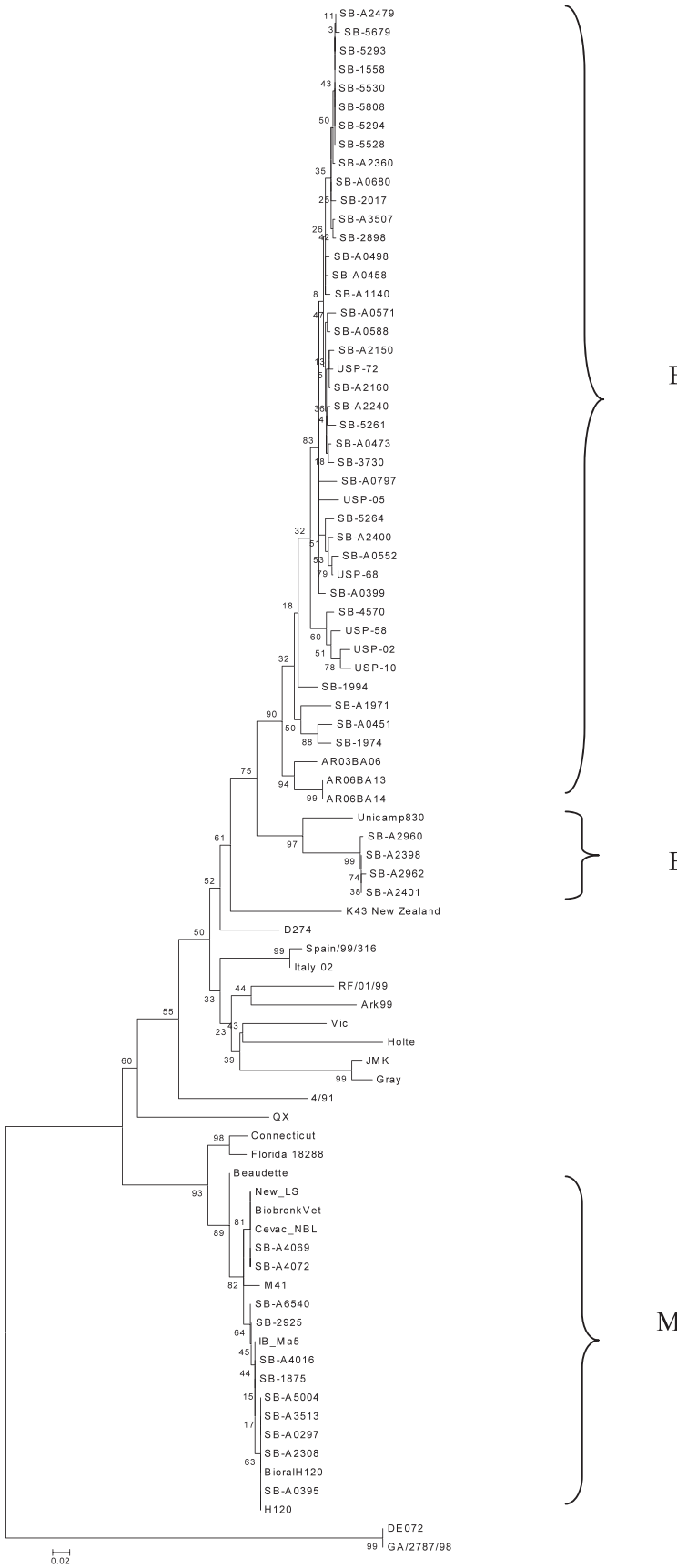

Fig. 1. Phylogenetic relationships of the Brazilian IBV samples (SB, USP, and UNICAMP) and some reference strains based on S1 partial nt sequences determined using MEGA 5.0 with the Clustal W method. Numbers along the branches refer to bootstrap values.

Table 2. Similarity analysis of nt and deduced amino acid sequences from the S1 gene of Brazilian isolates and some reference strains.

	Percent amino acid similarity											
Sequence	SB-A2400	SB-A0451	SB-A0473	SB-A1971	SB-1974	SB-1994	SB-4570	SB-5293	SB-5528	SB-5530	SB-5679	SB-A2962
SB-A2400	—	89.4	96.3	91.0	89.9	91.0	92.6	95.2	94.7	94.8	93.2	88.9
SB-A0451	92.8	—	89.3	91.5	95.8	89.9	90.5	89.4	88.8	89.0	87.8	84.1
SB-A0473	97.5	93.0	—	90.3	88.2	90.9	93.6	96.3	96.4	96.5	94.5	88.2
SB-A1971	92.6	93.6	92.5	—	92.0	89.9	89.4	89.9	88.7	88.9	87.1	86.7
SB-1974	92.2	95.8	92.0	94.5	—	89.9	88.9	87.8	87.0	87.2	85.0	84.1
SB-1994	92.1	92.1	92.5	92.2	93.5	—	92.1	89.9	89.3	90.1	87.8	84.1
SB-4570	93.5	93.3	93.8	91.7	91.7	93.5	—	91.5	91.1	90.7	89.1	86.2
SB-5293	97.0	92.8	98.0	92.4	92.2	92.4	92.9	—	100.0	100.0	98.0	87.3
SB-5528	96.7	92.1	98.4	91.7	91.6	92.5	92.3	100.0	—	100.0	98.0	85.8
SB-5530	96.7	92.3	98.4	91.8	91.7	92.6	92.3	100.0	100.0	—	97.9	86.0
SB-5679	96.4	91.6	97.7	90.5	90.7	91.8	91.2	99.3	99.3	99.3	—	83.7
SB-A2962	88.0	85.5	88.1	86.5	85.9	84.5	85.4	87.8	86.8	87.0	85.3	—
SB-A2960	88.2	85.7	88.2	86.7	85.9	84.7	85.5	88.0	87.0	87.2	85.5	99.6
SB-A2398	88.2	85.7	88.2	86.7	86.1	84.7	85.5	88.0	87.0	87.2	85.5	99.6
SB-A2401	88.2	85.7	88.2	86.7	86.1	84.7	85.5	88.0	87.0	87.2	85.5	99.8
USP-02	95.2	94.5	94.8	92.6	92.6	93.8	96.5	94.9	94.3	94.4	93.7	86.4
USP-05	94.4	91.2	94.7	91.1	89.8	90.5	90.7	94.5	94.7	94.4	94.6	86.1
USP-10	94.7	93.7	94.3	92.2	91.9	92.9	95.4	94.0	93.3	93.4	92.5	86.2
USP-68	98.5	92.9	98.3	92.5	92.6	92.8	93.5	98.0	97.8	97.9	97.5	88.1
USP-72	97.6	92.8	99.2	92.0	91.7	92.5	92.8	98.9	98.8	98.8	98.2	87.8
H120	73.3	72.9	74.3	73.3	73.4	73.3	72.3	73.3	72.1	72.8	71.2	72.0
4/91	72.1	71.7	72.9	72.3	71.9	72.8	72.8	72.1	71.5	71.7	70.2	71.7
D274	79.2	79.6	79.7	80.2	80.7	79.2	79.1	80.1	80.2	80.4	81.3	75.8
M41	73.3	72.9	73.4	73.8	73.8	73.3	72.7	73.6	72.5	73.2	71.4	72.7
QX	70.1	71.0	70.7	70.1	71.2	70.8	71.2	70.8	71.7	71.7	69.5	69.7
	Percent nucleotide similarity											

The prevention of IB is performed with the use of inactivated vaccines or, more frequently, attenuated live vaccines. Flock immunization is carried out using only vaccines from the Mass serotype in Brazil. However, there is a low identity among the S1 of Mass and the variant strains already identified in Brazil (7). Further, evidence suggests that vaccination with the Mass serotype provide inadequate protection against the variant strains that circulate in the region (10).

This study analyzed the nucleotide (nt) and the corresponding amino acid sequences of an IBV S1 gene variable region of samples obtained from different chicken flocks in the main poultry-producing regions from Brazil in recent years (2010 and 2011). The results were compared with IBV sequences of vaccine strains and field samples obtained from genetic databases of studies performed in South America and other continents.

MATERIALS AND METHODS

Field samples and vaccines. In the period from May 2010 to June 2011, samples of chicken tissues and organs (tracheas, lungs, kidneys, and caecal tonsils) were obtained from 60 different farms of the main poultry-producing regions of Brazil (Table 1). All these field samples were from broiler, breeder, and layer flocks with IB clinical signs and positive for IBV by nested reverse transcription- (RT-) PCR. In addition, the following five vaccines were provided by the suppliers: New LS Mass I® (Fort Dodge Saúde Animal Ltda, Campinas, SP, Brazil); CEVAC® NB L (Ceva Saúde Animal Ltda, Paulínia, SP, Brazil); Bio Bronk Vet® H-120 Laboratório Biovet, Vargem-Grande Paulista, SP, Brazil); and BIORAL® H-120 (Merial Saúde Animal Ltda, Campinas, SP, Brazil); Nobilis® IB Ma5 (MSD Saúde Animal, São Paulo, SP, Brazil).

RNA extraction. The total RNA of the clinical samples and viral vaccines was purified by a standard silica/guanidine isothiocyanate- (GuSCN-) based procedure (3) using a commercial kit (Newgene, Simbios Biotecnologia, Cachoeirinha, Rio Grande do Sul, Brazil). Briefly, all swab samples were resuspended on 2.5 mL of lysis buffer (GuSCN 5 M, Tris-HCl 0.1 M, EDTA 0.5 M, and Triton X-100) and incubated at 60 C for 10 min. Afterwards, 500 µl was transferred to a new tube, and 20 µl of silica suspension was added and mixed. Tubes were centrifuged at 8609 × *g* for 30 sec. The pellet was washed once with 500 µl and once with 150 µl of the washing buffer (GuSCN 5 M and Tris-HCl 0.1 M), twice with 150 µl of 75% ethanol and once with 150 µl of absolute ethanol. Silica suspension was dried at 60 C for 15 min. RNA was eluted with 50 µl of Tris-ethylenediaminetetraacetic acid buffer and incubated at 60 C for 5 min, and the solution was separated of the silica particles, centrifuging at 8609 × *g* for 3 min.

Nested RT-PCR. Amplification was performed in two consecutive reactions. In the first one, the RT-PCR was carried out in a total volume of 25 µl, using 2 µl of RNA template, 16 µl of H_2O, 0.6 µl of dithiothreitol (0.1 M), 0.7 µl of deoxynucleotide triphosphate (2.1 mM of deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate), 0.2 µl of the each primer (50 µM) S15 (14) and CK2 (13), 0.1 µl of RNAguard 40 U/µl (GE Healthcare, Fairfield, CT), 0.1 µl of Moloney Murine Leukemia Virus reverse transcriptase 200 U/µl (Promega, Madison, WI), 0.2 µl of Taq DNA polymerase (5 U/µl) and 5 µl of a 5× concentrate RT and Taq DNA polymerase buffer (Cenbiot Enzimas, Porto Alegre, Rio Grande do Sul, Brazil). Amplification was performed in a Veriti 96 Thermo Cycler (Applied Biosystems Inc., Norwalk, CT) with the following conditions: one cycle at 37 C for 30 min and 35 cycles at 94 C for 20 sec, 50 C for 40 sec, and 72 C for 1 min. The second PCR (nested) was carried out in a volume of 50 µl using 2 µl of amplified DNA, 39.1 µl of H_2O treated, 1.5 µl of $MgCl_2$ (50 mM), 1.5 µl of deoxyribonucleotide triphosphate (2.1 mM), 0.2 µl of each primer (50 µM) IBVS1-1F (5′-TTR TTR RTW AGA GAT GTT GG-3′) and IBVS1-3R (5′-GCT TTR AAR TRA ACA CCT GC-3′), 0.2 µl of Taq DNA polymerase (5 U/µl) and 5 µl of a 10× concentrate Taq DNA polymerase buffer (Cenbiot Enzimas). The primers for the second amplification were newly designed for the study. Thermal cycling was performed in the same equipment with the following steps: one cycle at 94 C for 3 min, 35 cycles at 94 C for 20 sec, 55 C for 40 sec and 72 C for 1 min, and a final

Table 2. Extended.

Percent amino acid similarity												
SB-A2960	SB-A2398	SB-A2401	USP-02	USP-05	USP-10	USP-68	USP-72	H120	4/91	D274	M41	QX
89.4	89.4	89.4	94.2	92.6	93.1	96.6	95.5	71.4	71.7	79.2	70.3	67.9
84.7	84.7	84.7	91.0	88.9	88.4	89.4	88.7	70.3	70.7	78.1	68.1	67.9
88.8	88.8	88.8	93.6	92.5	92.0	96.6	97.7	71.4	72.0	80.1	69.2	68.7
87.2	87.2	87.2	91.0	89.9	88.8	90.4	89.2	71.3	73.2	79.1	70.2	68.3
84.7	84.7	84.7	89.9	87.8	88.9	88.8	87.0	70.3	71.2	78.1	68.7	67.9
84.7	84.7	84.7	92.1	89.9	89.9	91.6	91.0	69.8	71.7	78.7	68.7	69.6
86.8	86.8	86.8	94.2	90.5	92.1	92.7	92.1	70.9	73.4	79.8	69.8	69.0
87.8	87.8	87.8	94.2	92.1	92.1	97.2	97.7	70.3	70.7	80.3	69.2	69.0
86.4	86.4	86.4	93.5	91.7	91.1	97.0	97.6	69.8	70.1	80.4	68.5	70.1
86.6	86.6	86.6	93.6	91.3	91.3	97.1	97.7	70.3	70.1	80.1	69.1	70.1
84.4	84.4	84.4	91.8	91.2	89.1	95.2	95.2	66.4	69.0	79.4	65.0	67.6
98.9	98.9	99.5	88.4	86.2	87.8	87.7	87.0	68.7	68.5	74.9	68.1	66.3
—	98.9	99.5	88.9	86.8	88.4	88.3	87.6	68.7	69.0	75.4	69.2	66.3
99.6	—	99.5	88.9	86.8	88.4	88.3	87.6	69.2	68.5	75.4	68.7	66.3
99.8	99.8	—	88.9	86.8	88.4	88.3	87.6	69.2	69.0	75.4	68.7	66.3
86.6	86.6	86.6	—	92.1	95.8	94.4	92.7	71.4	72.3	79.8	69.8	69.0
86.2	86.2	86.2	92.8	—	89.4	92.2	92.1	69.8	70.1	77.0	68.7	68.5
86.4	86.4	86.4	98.4	92.3	—	92.2	90.4	71.4	72.3	78.7	69.8	68.5
88.3	88.3	88.3	95.2	94.8	94.3	—	97.7	70.3	72.4	80.3	70.3	70.1
87.9	87.9	87.9	94.4	94.9	93.4	98.7	—	71.2	70.9	79.5	71.2	69.2
72.2	72.2	72.2	72.7	72.7	72.5	72.8	72.9	—	67.6	68.7	93.4	73.1
71.7	71.7	71.9	72.6	72.3	72.6	72.1	72.1	74.0	—	71.8	66.5	69.6
76.0	76.0	76.0	79.2	78.5	79.2	79.7	79.1	75.1	73.8	—	67.0	70.7
72.9	72.9	72.9	72.9	72.7	72.7	73.6	73.7	96.3	74.2	74.6	—	73.1
69.6	69.6	69.6	71.0	71.2	70.8	71.6	70.9	75.6	74.5	74.3	75.5	—
Percent nucleotide similarity												

extension cycle at 72 C for 5 min. Nested RT-PCR amplified 590-bp products corresponding to the IBV S1 region. The PCR products were run on polyacrylamide gel electrophoresis and stained with silver nitrate.

Sequencing and analysis. The amplified PCR products were sequenced using sense and antisense PCR primers with Sequencer ABI 130 3130xl Genetic Analyzer (Applied Biosystems Inc.). The nt sequences from both strands were edited, assembled, and analyzed using the Clustal W method available in the Bioedit software package (version 7.0.3.0. available at: http://www.mbio.ncsu.edu/bioedit/bioedit). All the Brazilian and reference nt sequences were compared using the neighbor-joining method with 1000 bootstrapping replicates for phylogenetic analysis and presence of insertions and deletions (MEGA software version 5.0; available at: http://www.megasoftware.net/). Amino acid sequences were also aligned using Clustal W method and compared for similarity analysis. These sequences were further evaluated using both the NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc (http://www.cbs.dtu.dk/services/NetOGlyc/) servers, searching for any glycosylation modification derived by the insertion or loss of amino acids.

Nt and amino acid sequences of reference and South American strains used in the analysis were retrieved from GenBank database. The sequences were 4/91 (AF093794), Ark99 (L10384), AR03BA06 (FJ167386), AR06BA13 (FJ167376), AR06BA14 (FJ167375), Beaudette (X02342), Conn (EU283057), D274 (X15832), DE072 (EU359658), Florida 18288 (AF027512), GA-2787-98 (AF274438), Gray (L18989), H120 (M21970), Holte (L18988), Italy-02 (AJ457137), JMK (L14070), K43 New Zealand (AF151958), M41 (AY851295), QX (AF193423), RF.01.99 (AJ440783), Spain-99-316 (DQ064809), USP-02 (DQ448273), USP-05 (DQ492308), USP-10 (DQ448275), USP-58 (GU383095), USP-68 (GU383105), USP-72 (GU383109), Unicamp830 (HM561897), and Vic (DQ490221).

RESULTS

IBV screening and partial amplification of S1 gene. The 60 field samples resulted in 51 positive for IBV S1 nested RT-PCR, displaying the expected amplified fragment of approximately 590 bp. The five vaccine strains also presented an amplified fragment with approximately the same length. The 51 amplification products were sequenced and gave rise to 49 sequences with good quality. The nt and amino acid sequences data are available in GenBank (Table 1).

Analysis of the nt sequences. IBV S1 sequences obtained in the present study were compared with reference and variant strain sequences from different regions of the world retrieved from GenBank. The similarity between S1 gene fragments ranging from 438 to 569 bases in length was evaluated (Table 2), and a phylogenetic tree was constructed (Fig. 1). In general, the Brazilian field samples and vaccine strains sequenced in the present study clustered in two groups/branches: 1) the five vaccine and 11 field samples that clustered with Mass serotype reference strains (Beaudette, M41 and H120); 2) the other 38 field samples that clustered with several local variant strains previously characterized in studies performed in Brazil and Argentina (7,12,16,18).

The first group (identified as Mass genotype) showed nt identities, ranging from 96.3% to 100% among themselves and reference strains M41 and H120 (data not shown). Sequences of the other cluster presented a higher diversity, varying from 84.5% to 100% of identity. The lowest sequence identity was shared by samples IBV/BRAZIL/2010/SB-1994 and IBV/BRAZIL/2010/SB-A2962, while the highest one was found between three samples (IBV/BRAZIL/2010/SB-5293, IBV/BRAZIL/2010/SB-5528, and IBV/BRAZIL/2010/SB-5530; Table 2). However, they are well separated in two genotypic clusters in the phylogenetic tree: one with 34 samples similar to the previously described BR-I genotype (7) and the other with four samples with similarity to the previously characterized variant IBV/BRAZIL/2008/UNICAMP830 (12).

Among the 34 samples from the BR-I genotype, 29 performed a specific and homogenous group in the phylogenetic tree with a high nt sequence identity among themselves (95.4%–100%) and to the

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SB-A2398  VVFVTHCFKHGSTECPLTGLIPSGHIRIAAMKNNGTGPSDLFYNL
SB-A2401  VVFVTHCFKHGSTECPLTGLIPSGHIRIAAMKNNGTGPSDLFYNL
SB-1974   VVFVTHCYKRGAIECPLTGLIPQNHIRISVMKKGNTGPSGLFYNL
SB-1994   VVFVTHCYKSGSGECPLTGLIPQNHIRISAMKIGNTGPSGLFYNL
SB-4570   VVFVTHCYKSGSGECPLTGLIPQNHIRISAMKTGNTGPSGLFYNS
SB-5808   VVFVTHCYKSGSTACPLTGLIPQNHIRISAMKQGNNGPSGLFYNL
SB-5293   VVFVTHCYKSGSTACPLTGLIPQNHIRISAMKQGNNGPSGLFYNL
SB-A1971  VVFVTHCYKSGQSECPLTGLIPQNHIRISAMKGGNTGPSGLFYNL
USP-02    VVFVTHCYKSGSTECSLTGLIPQNHIRISAMKPGNTGPSGLFYNL
USP-10    VVFVTHCFKRGSNECPLTGFITQNHIRISAMKQGNTGPSSLFYNL
4-91      TVFVTHCFKSQQGSCPLTGMIPQNHIRISAMRSG-----FLFYNL
D274      VVFVTHCYKSSHGSCPLTGLIPQNHIRISAMKNS-----SLFYNL
QX        TVFVTHCYSSGSGSCPITGMIPRDHIRISAMKNG-----SLFYNL
H120      TVFVTHCYKHVG--CPITGMLQQHSIRVSAMKNG-----QLFYNL
M41       TVFVTHCYKYDG--CPITGMLQKNFLRVSAMKNG-----QLFYNL
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Fig. 2. Alignment of the amino acid partial sequence of the S1 gene (amino acid 108–145, reference strain H120) showing the amino acid insertions (in bold).

previously characterized Brazilian IBV samples of the BR-I genotype (IBV/BRAZIL/2005/USP-02, IBV/BRAZIL/2005/USP-05, IBV/BRAZIL/2005/USP-10, IBV/BRAZIL/2009/USP-68, and IBV/BRAZIL/2009/USP-72). The great majority of the IBV variants from this group clustered in a specific branch with IBV/BRAZIL/2009/USP-72, a sample isolated from a poultry flock of the Paraná state (Brazilian southern region) in 2009 (7). The other five samples did not cluster to any other previously identified IBV variant, but three of them (IBV/BRAZIL/2010/SB-1974, IBV/BRAZIL/2010/SB-A0451, and IBV/BRAZIL/2010/SB-A1971) grouped together and presented a high nt similarity (93.5%–95.8%). These last samples and IBV/BRAZIL/2010/SB-1994 were identified in flocks from the northeastern region (Alagoas and Bahia states) and presented similarity with IBV variants previously characterized in Argentina (16).

The remaining four samples (IBV/BRAZIL/2010/SB-A2398, IBV/BRAZIL/2010/SB-A2401, IBV/BRAZIL/2010/SB-A2960, and IBV/BRAZIL/2010/SB-A2962) clustered together. All of these four samples were identified in flocks from the midwestern region (Mato Grosso State) and presented a very high identity (99.6%–99.8%). Further, these samples presented less than 89% of nt identity with all other sequences, including the other Brazilian variant sequences characterized here and in previous studies, with the exception of one IBV sample identified as IBV/BRAZIL/2008/UNICAMP830 collected in the southeastern region in 2008 (12). This sequence presented (91.3%) of nt similarity with these four samples and consequently was more closely related with this group in the phylogenetic tree. The IBV variants that grouped within this genotypic cluster (the four samples of the midwestern region and IBV/BRAZIL/2008/UNICAMP830) were referred to as the Brazil-II (BR-II) genotype (Fig. 1).

A phylogenetic tree constructed using only the nt sequences of the hypervariable region 1/2 (HVR1/2 [15]) showed a similar topology (data not shown). Sequences were also analyzed for nt insertions or deletions in the S1 gene. All Brazilian IBV variants presented 21 additional nt when compared with Mass reference strains (H120, M41, and Beaudette), vaccine strains, and Brazilian IBVs of the Mass group. Two nt insertions were observed in the alignment and were located at positions 358–359 (6 nt) and 418–419 (15 nt) of H120 reference strain.

Analysis of the amino acid sequences. The similarities among the partial S1 amino acid sequences (corresponding to the 4th to 186th residues of the H120 strain) were also evaluated. A high similarity within all the Brazilian Mass field samples was observed (95.1%–100%). On the other hand, variant strains presented a higher S1 amino acid diversity than IBVs of the Mass group (84.1%–100% similarity), with the lowest sequence identity observed between samples IBV/BRAZIL/2010/SB-1974, IBV/BRAZIL/2010/SB-1994, IBV/BRAZIL/2010/SB-A0451, and IBV/BRAZIL/2010/SB-A2962 (Table 2). An amino acid similarity of 85% to 100% was observed among the 34 samples of the first Brazilian ''variant'' group, while a high similarity (98.9%–99.5%) was observed among the four samples of the second group. In the comparison between samples of the two groups, a maximum of 89.4% of amino acid identity was observed (Table 2). All Brazilian variants were also compared with H120 and M41 reference sequences, and similarity values ranged from 65% (IBV/BRAZIL/2010/SB-5679) to 71.4% (IBV/BRAZIL/2011/SB-A0473; Table 2).

As expected, a higher amino acid sequence diversity was observed in the HVR1/2 in the comparison among all samples of the two Brazilian genotypes BR-I and BR-II (75.5% to 100% of identity), with the lowest similarity observed between samples IBV/BRAZIL/2010/SB-1974 and IBV/BRAZIL/2010/SB-A2962 (data not show). An amino acid homology of 80.9% to 100% was observed among the 34 samples of the first Brazilian ''variant'' group, while a high similarity (97.9%–98.9%) was observed among the four samples of the second group. All Brazilian variants were also compared with Mass serotype vaccine strain H120 sequences, and similarity values ranged from 56.8% to 63.2% in this variable region (data not shown).

In the nt sequence alignments, specific substitutions and fragments insertions were observed. Specific one nt substitutions generated eight specific amino acid residues changes in all sequences of the group BR-II when compared with BR-I sequences: T66R, Y115F, Q130S, N131G, Q140N, G141N, N142G, and G147D. Interestingly IBV/BRAZIL/2008/UNICAMP830 is similar to BR-II in seven of these positions (exception to 140: Q140T), but we could not evaluate the first 60 amino acid residues (IBV/BRAZIL/2008/UNICAMP830 available sequence is shorter). Furthermore, two nt fragments insertions allowed the introduction of a variable amino acid insertion of two residues for the first position and another more conserved motif of five amino acid residues (NTGPS) for the second position in all Brazilian variants in comparison to Mass strains. The second insertion was found in the final of the HVR1/2 of the S1 protein (15) and the last three amino acids (glycine, proline, and serine) were conserved in all Brazilian IBV variants analyzed in the present and previous studies (Fig. 2) (12,16,18). These insertions did not change the predicted glycosilation pattern (evaluated with NetNGlyc and NetOGlyc servers).

DISCUSSION

IBV is present in the majority of the regions where poultry is reared and spreads fast in nonprotected birds. The high frequency of genetic mutations and recombination events contribute to the emergence of new strains and serotypes of the virus. Some of these IBVs remain confined in one region, while others have a worldwide distribution (8).

Few IBV strains (as, for example, Mass, 4/91, and, more recently, QX) appear to have spread to different regions of the world. Among these strains, here we describe 11 flocks with the Mass genotype in different states of Brazil in the years 2010 and 2011. Mass strains have been detected in commercial poultry flocks since 1957 in Brazil (17). The intensive use of live vaccines with Mass strains (the only vaccine serotype allowed in Brazil) has probably spread this specific serotype in all poultry-producing regions of the country. It is possible that the isolates clustered in the Mass genotype were from vaccine origin; however, it was not possible to determine if these

specific flocks were previously vaccinated, although the use of live attenuated vaccines is a frequent procedure in poultry commercial flocks and the Mass serotype strains H120 and H52 are indeed widely used as vaccine viruses in Brazil. On the other hand, we did not find samples that grouped with strains from other regions of the world, including the ones present in different continents (as, for example, 4/91 and QX). Previous studies showed the rare occurrence of IBV strains from Conn and 4/91 groups in the Brazilian southeastern region (12,19). One explanation for these findings could be the use of imported live vaccines with these strains, as previously proposed (12).

Despite the large use of live vaccines with Mass strains, here we describe the predominance of a specific group of Brazilian IBV variants. These IBV variants are significantly different from the Mass strains, presenting a low nt and amino acid similarity. Previous studies have already shown that the majority of the variant strains found in Brazil clustered in the phylogenetic analysis, using partial characterization of the N (1) or the S genes (12,18). Two recent studies demonstrated that these variant viruses were present in different states of the country over a 7-yr period (2003 to 2009) and were referred as Brazil (19) and BR-I genotype (7). The majority (89.5%) of the Brazilian IBV variants described in the present study clustered with the previously characterized BR-I genotype strains, showing that this genotype appears to have spread more than any other IBV variant in the last years predominating in the main poultry producing regions of the country (south and southeast). Furthermore, other Brazilian variant sequences previously characterized as D207 group (12) also clustered with these samples (data not shown). Inside this cluster, one subgroup of four strains is slightly divergent and includes IBV samples found in chicken flocks from the northeastern region that are similar to isolates from Argentina (16). In the midwestern region of the country, another group of four samples presented a more significant divergence from the previous IBV variants characterized in Brazil. This group was similar with only one IBV sample previously identified in Brazil (IBV/BRAZIL/2008/UNICAMP830), collected in the southeastern region in 2008 (12). As this group presented nt and amino acid diversity higher than 10% in comparison to samples of the BR-I genotype, we suggest the emergence of novel IBV variants and proposed they should be classified in a new genotype: BR-II. This new genotype appears to have the same origin of the BR-I, and the observed divergence is probably related to a regional dissemination of this genotype (the midwest is a growing and independent poultry-producing region). Further, these results showed a higher IBV diversity than previous studies, demonstrating that Brazilian variant genotypes are not as stable over time as previously described (7). A broader epidemiologic study should be conducted to understand the IBV spread in all Brazilian poultry-producing regions.

The majority of the IBV epidemiologic studies analyze the S1 gene because this is a highly variable genomic region, and the deduced amino acid sequence can be an important predictor of immune status in chickens (10,15). It was already demonstrated that 1) there is a strong correlation between the S1 sequence, mainly in HVR1/2, and protective relatedness values obtained in virus neutralization tests (15) and 2) Brazilian variant IBVs have more than 25% of divergence from Mass vaccine strains in the S1 amino acid sequence and, consequently, a low antigenic relationship by virus neutralization (7). In the present study, we confirmed this high divergence in the S1 amino acid sequence and observed eight specific substitutions between BR-I and BR-II sequences and, further, two characteristic nt insertions in all Brazilian variant IBVs. These insertions are responsible for seven additional amino acid residues in the S1 polypeptide chain when compared with the Mass genotype. One of these insertions occurs in the final of the HVR1/2 and is conserved among all the Brazilian variant IBVs, with three common residues: glycine, proline, and serine. These insertions were also observed in some IBV variants from other regions of the world, but not in the classical Mass strains (data not shown). Although the specific location of the receptor-binding domain within S1 is not known for IBV and varies among other coronaviruses, monoclonal antibody analyses revealed that some of the amino acids from the virus-neutralizing epitopes are located within the HVR1/2 (15,20). This is very suggestive that these insertions are probably responsible for a different three-dimensional structure providing Brazilian variant IBVs with a selective advantage in chicken flocks vaccinated with heterologous serotypes such as Mass (5). Other possible effects of these insertions were further analyzed using heterologous recombination, but no N or O glycosilation sites or other common modifications could be found. The construction of a three-dimensional model was also not possible due the lack of appropriate templates. These results could explain a possible failure of Mass strains to control the dissemination of these Brazilian variant IBVs.

On the other hand, S1 glycoprotein is also an important epitope in adsorption to the cellular receptor and virus entry into the host cell. Amino acid divergences in the peptide chain were found in strains with different cell tropisms (4,5,20). The same modifications in the S1 three-dimensional structure could be affecting cell tropism of the Brazilian variant IBVs. Although there is evidence that the same genotype can be associated to different cell tropisms and could be related with multiple clinical conditions (7), new studies should be performed to define the specific location and importance of this region for the development of the disease.

In summary, this study confirms the high prevalence of one main IBV genotype (BR-I) in commercial poultry flocks from Brazil in recent years (2010 and 2011). However, we found a new group of variant IBV in Brazil (BR-II) in the midwestern region, a growing poultry-producing center. It was also demonstrated that all Brazilian IBV variants (BR-I and BR-II genotypes) presented specific nt and amino acid sequence insertions in the S1 gene in comparison to the Mass vaccine strains that could be associated with the occurrence of clinical disease in vaccinated flocks.

REFERENCES

1. Abreu, J. T., M. M. Mourão, C. E. Santos, C. J. M. Veloso, J. S. Resende, R. B. Flatschart, A. V. Folgueras-Flatschart, S. N. Júnior, M. M. Santoro, A. C. R. Mendes, G. R. Franco, A. Silva, A. B. Campos, and S. Fernandez. Molecular studies of the Brazilian infectious bronchitis virus isolates. Rev. Bras. Cienc. Avic. 12:107–110. 2010.

2. Alvarado, I. R., P. Villegas, N. Mossos, and M. W. Jackwood. Molecular characterization of avian infectious bronchitis virus strains isolated in Colombia during 2003. Avian Dis. 49:494–499. 2005.

3. Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-Van Dillen, and J. Van Der Noordaa. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28:495–503. 1990.

4. Casais, R., B. Dove, D. Cavanagh, and P. Britton. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J. Virol. 77:9084–9089. 2003.

5. Cavanagh, D. Coronavirus avian infectious bronchitis virus. Vet. Res. 38:281–297. 2007.

6. Cavanagh, D., and J. Gelb Jr. Infectious bronchitis. In: Diseases of poultry, 12th ed. Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne, eds. Iowa State University Press, Ames, IA. pp. 117–135. 2008.

7. Chacón, J. L., J. N. Rodrigues, M. S. Assayag Júnior, C. Peloso, A. C. Pedroso, and A. J. P. Ferreira. Epidemiological survey and molecular characterization of avian infectious bronchitis virus in Brazil between 2003 and 2009. Avian Pathol. 40:153–162. 2011.

8. Cook, J. K. A., M. Jackwood, and R. C. Jones. The long view: 40 years of infectious bronchitis research. Avian Pathol. 41:239–250. 2012.

9. De Wit, J. J., J. K. A. Cook, and H. M. J. Van Der Heijden. Infectious bronchitis virus variants: a review of the history, current situation and control measures. Avian Pathol. 40:223–235. 2011.

10. De Wit, J. J., W. A. J. M. Swart, and T. H. F. Fabri. Efficacy of infectious bronchitis virus vaccinations in the field: association between the α-IBV IgM response, protection and vaccine application parameters. Avian Pathol. 39:123–131. 2010.

11. Di Fábio, J., and L. Y. B. Buitrago. Bronquite infecciosa das galinhas. In: Doenças das aves, 2nd ed. A. Berchieri Jr, E. N. Silva, J. Di Fábio, L. Sesti, and M. A. F. Zuanaze, eds. Facta, Campinas, Brasil. pp. 631–648. 2009.

12. Felippe, P. A. N., L. H. A. Silva, M. M. A. B. Santos, F. R. Spilki, and C. W. Arns. Genetic diversity of avian infectious bronchitis virus isolated from domestic chicken flocks and coronaviruses from feral pigeons in Brazil between 2003 and 2009. Avian Dis. 54:1191–1196. 2010.

13. Keeler, C. L. J., K. L. Reed, W. A. Nix, and J. J. Gelb. Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S-1) gene. Avian Dis. 42:275–284. 1998.

14. Kwon, H. M., M. W. Jackwood, and J. J. Gelb. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis. 37:194–202. 1993.

15. Ladman, B. S., A. B. Loupos, and J. Gelb Jr. Infectious bronchitis virus S1 gene sequence comparison is a better predictor of challenge of immunity in chickens than serotyping by virus neutralization. Avian Pathol. 35:127–133. 2006.

16. Rimondi, A., M. I. Craig, A. Vagnozzi, G. König, M. Delamer, and A. Pereda. Molecular characterization of avian infectious bronchitis virus strain from outbreaks in Argentina (2001–2008). Avian Pathol. 38:149–153. 2009.

17. Silva, E. N. Infectious bronchitis in Brazilian chickens: current data and observations of field service personnel. Rev. Bras. Cienc. Avic. 12:197–203. 2010.

18. Villarreal, L. Y. B., P. E. Brandão, J. L. Chacón, A. B. S. Saidenberg, M. S. Assayag, R. C. Jones, and A. J. P. Ferreira. Molecular characterization of infectious bronchitis virus strains isolated from the enteric contents of Brazilian laying hens and broilers. Avian Dis. 51:974–978. 2007.

19. Villarreal, L. Y. B., T. L. Sandri, S. P. Souza, L. J. Richtzenhain, J. J. De Wit, and P. E. Brandão. Molecular epidemiology of avian infectious bronchitis in Brazil from 2007 to 2008 in breeders, broilers, and layers. Avian Dis. 54:894–898. 2010.

20. Weiss, S. R., and S. Navas-Martin. Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. Microbiol. Mol. Biol. Rev. 69:635–664. 2005.

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