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Molecular Analysis of Brazilian Infectious Bronchitis Field Isolates by Reverse Transcription–Polymerase Chain Reaction, Restriction Fragment Length Polymorphism, and Partial Sequencing of the N Gene

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SUMMARY. Molecular analysis of 15 Brazilian infectious bronchitis virus (IBV) isolates, obtained from clinical outbreaks of the disease in chickens (broilers or layers) in the state of Minas Gerais (Brazil) between 1972 and 1989, is reported. Using the N protein gene as target, IBVs were analyzed by reverse transcription–polymerase chain reaction/restriction fragment length polymorphism (RT-PCR/RFLP) with the restriction enzymes *Ava*II, *Hph*I, *Sau*96I, and *Tsp*509I and cDNA sequencing. Results obtained from those isolates were compared to 19 sequences available in GenBank. N gene RFLP profiles, cDNA sequences, and predicted amino acid composition were used for the construction of dendrograms. Brazilian isolates were grouped into one distinct group. Identity of predicted N protein amino acid composition varied from 45% (between isolates G and 208) up to 99% (PM1 and PM2), and, when compared to the other IBVs, the amino acid identity was from 42% (Q3/88 and G) up to 97% (D41 and PM1). The great genetic diversity was shown to occur before the official use of vaccination in Brazil and has remained thereafter.

RESUMEN. Análisis molecular de aislamientos del virus de bronquitis infecciosa provenientes de Brasil mediante reacción en cadena por la polimerasa-transcriptasa reversa, análisis del polimorfismo de la longitud de los fragmentos de restricción y secuenciación parcial del gen N.

Se reporta el análisis molecular de 15 aislamientos del virus de bronquitis infecciosa obtenidos de brotes clínicos de la enfermedad en aves (pollos de engorde o ponedoras) en el estado de minas Gerais (Brasil), entre los años 1972 y 1989. Los virus de bronquitis infecciosa fueron analizados mediante la reacción en cadena por la polimerasa-transcriptasa reversa, el análisis del polimorfismo de la longitud de los fragmentos de restricción utilizando las enzimas de restricción *Ava*II, *Hph*I, *Sau*96I y *Tsp*509I y mediante secuenciación parcial del gen N. Los resultados obtenidos de estos aislamientos se compararon con 19 secuencias disponibles en el banco de genes (GenBank). Para la construcción de dendogramas, se utilizó el patrón del análisis del polimorfismo de la longitud de los fragmentos de restricción, las secuencias del ADN complementario y la secuencia estimada de aminoácidos. Los aislamientos brasileños se agruparon en un grupo distinto. La similitud de la composición estimada de aminoácidos varió desde 45% (entre los aislamientos G y 208) hasta 99% (PM1 y PM2) y cuando se compararon con otros virus de la bronquitis infecciosa, la similitud de la composición estimada de aminoácidos fue desde 42% (Q3/88 y G) hasta 97% (D41 y Pm1). Se demostró que la gran diversidad genética ocurría antes del uso oficial de la vacunación en Brasil y ha permanecido desde entonces.

Key words: infectious bronchitis virus, coronavirus, nucleoprotein, phylogenetic analysis, molecular epidemiology

Abbreviations: AF = allantoic fluid; Ark = Arkansas; ATCC = American Type Culture Collection; Conn = Connecticut; IB = infectious bronchitis; IBV = infectious bronchitis virus; M = transmembrane protein; Mass = Massachusetts; N = nucleocapsid; NDV = Newcastle disease virus; RE = restriction enzyme; RFLP = restriction fragment length polymorphism; RT-PCR = reverse transcription–polymerase chain reaction; S1 = S1 subunit spike glycoprotein; SARS = severe acute respiratory syndrome; SPF = specific pathogen free; UV = ultraviolet

Infectious bronchitis (IB) is a global widespread highly contagious and economically important viral disease of chickens (4,5) caused by a coronavirus (infectious bronchitis virus [IBV]).

The occurrence of variant IBV is a consequence of mutations and recombinations between field isolates and vaccine strains and may be heightened in regions where nonuniform massive vaccination schedules are employed. Part of the difficulty in obtaining an efficient control through vaccination is due to the occurrence of variant isolates against which there are no cross-protections (2,4,9,14,30,45,46). For the adoption of adequate control measures, the actual profiles of local isolates should be known. The molecular analysis of IBV isolates may provide the necessary information for updating vaccines and vaccination strategies (4,8,18).

The N protein is involved in mechanisms of IBV replication regulation (13,21,44), grouping virus particles, providing partial genome protection (7,9,20), and inducing T and B cell mediated immune responses (24,34,35,36). The N protein coding gene is highly conserved (12,29,39,40) even through long time spans of 30–60 yr (28,33). In addition, the N gene sequence similarity allows grouping of serologically very distinct strains into large groups (27,47).

The characterization of IBV has been performed by analysis of reverse transcription–polymerase chain reaction (RT-PCR) products

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Table 1. IBV isolates of infectious bronchitis outbreaks in commercial chickens (Minas Gerais, Brazil).

Isolate	Number of passages in SPF eggs	Year of isolation	Type of chicken[A]	GenBank accession numbers	Clinical signs[B]
IBV/BR/208/1972	6	1972	L	DQ659353	R/ED/AEP
IBV/BR/TII/1975	6	1975	B	DQ659366	R
IBV/BR/G/1975	8	1975	B	DQ659361	R
IBV/BR/29-78/1978	6	1978	B	DQ659357	R
IBV/BR/200/1981	4	1981	B	DQ659352	R
IBV/BR/283/1983	3	1983	B	DQ659354	R+NN
IBV/BR/290/1983	3	1983	B	DQ659355	R
IBV/BR/297/1983	3	1983	B	DQ659356	R
IBV/BR/319/1983	3	1983	B	DQ659358	R+NN
IBV/BR/327/1983	3	1983	B	DQ659359	R+NN
IBV/BR/351/1984	4	1984	B	DQ659360	R+NN
IBV/BR/PM1/1987	4	1987	B	DQ659362	R
IBV/BR/PM2/1987	3	1987	B	DQ659363	R
IBV/BR/PM3/1989	3	1989	B	DQ659364	R
IBV/BR/PM4/1989	3	1989	B	DQ659365	R

[A]L, layer; B, broiler.
[B]R, respiratory; ED, egg drop; AEP, abnormal egg production; NN, nephritis–nephrosis.

through restriction fragment length polymorphism (RFLP) or sequencing, mainly of the S1 subunit spike glycoprotein (S1) protein coding gene (3,10,29). The S1 protein is involved in the induction of IBV neutralizing and serotype-specific antibodies (3,15) and shows the greatest variation among strains, as compared to the structural proteins nucleocapsid (N), transmembrane protein (M), and small hydrophobic transmembrane protein (18,26). RFLP and sequencing have been employed and are convenient methods for classifying IBV strains (10,25).

N gene conserved region primers have been designed (47) and evaluated subsequently for IBV detection (1,27). Slight differences observed on amplicons of serologically indistinguishable isolates may be useful for epidemiologic and phylogenetic studies. The analysis of the N protein coding gene might be useful for determining relationships between isolates, otherwise unnoticed by S1 analysis. It is expected that the N protein gene analysis might indicate tendencies in IBV evolution in chickens subject to strong IBV selective pressures, including specific immunity and vaccine virus recycling. The isolates were previously characterized (38) using monoclonal antibodies directed to N protein and S2 subunit spike glycoprotein conserved regions, and results showed differences among them. This paper aims to characterize 15 Brazilian IBV isolates using RFLP and sequence analysis of amplicons, in comparison to reference strains and published sequences.

MATERIALS AND METHODS

Viruses. Fifteen field isolates of IBV were recovered from natural outbreaks of IB in different types of commercial chicken flocks in Minas Gerais, Brazil (Table 1). Tissue samples were obtained from trachea, lungs, air sacs, or kidneys of nonvaccinated broiler or layer flocks between 1972 and 1989.

The La Sota strain of Newcastle disease virus (NDV) and pooled allantoic fluid (AF) of noninfected specific pathogen free (SPF) eggs were used as negative controls. Three reference strains (M41, Connecticutt-A5968 [Conn-A5968], and Arkansas-99 [Ark-99], from the American Type Culture Collection [ATCC]) were used as PCR positive controls.

Virus propagation. Isolates and reference strains were propagated for 48–72 hr into the allantoic cavity of 9–11-day-old SPF eggs (16). The allantoic fluid was collected and the virus was concentrated using 45-mm diameter dialysis tubing with 12,000 Da cutoff (Spectrapor™, Spectrum Medical Industries, Taejon, Korea) against 50% (w/v) polyvinylpyrrolidone (PVP-360, Sigma Chemical Co., St. Louis, MO) for 24–48 hr at 4 C. Concentrates were diluted at 1:100 in sterile phosphate-buffered saline, pH 7.4.

RNA extraction. Viral RNA was prepared as described previously (1) with modifications. Briefly, 15 μL of ribonucleoside vanadyl complexes (Sigma Chemical Co.) were added to 500 μL of viral concentrate. RNA extraction was performed with Trizol LS (Invitrogen, Gaithersburg, MD) and chloroform, and the nucleic acid was precipitated with isopropanol and ethanol (both from Sigma Chemical Co.) and resuspended in 20 μL 18 MΩ RNase-free water. An aliquot of 10 μL was used for immediate reverse transcription (RT).

Oligonucleotides. The primers, designated IBVN1 (reverse) and IBVN2 (forward), were previously described by Zwaagstra *et al.* (47) and flank a 438 base sequence on the 5′ termini of the N gene.

RT-PCR. The RT was performed with SuperScript II RNase H^- (Gibco BRL, Sao Paulo, Brazil) according to the manufacturer and previous reports (1,30), with modifications. The amplification was conducted starting at 94 C for 3 min, 58 C for 2 min, 72 C for 3 min, and 28 cycles of 94 C for 1 min, 64 C for 2 min, and 72 C for 3 min. The final extension was at 72 C for 10 min. The analysis of the PCR products was performed by electrophoresis on ethidium bromide-containing 1% agarose gels –0.5× TBE under ultraviolet (UV) light.

RFLP. The M41 strain was used as *Ava*II (Promega, Madison, WI) restriction endonuclease (RE) positive control, employed for the digestion of isolates PCR amplicons, according to the manufacturer's instructions. Digested materials were resolved by electrophoresis (2% agarose with ethidium bromide) and visualized at UV. The N gene sequence (388 bases) was mapped for the relevant restriction sites using Webcutter 2.0 (19) and compared to the published data (GenBank). To design discriminatory RFLP, the sequence of each Brazilian isolate was submitted to virtual digestion using Webcutter 2.0 with *Ava*II, *Hph*I, *Sau*96I, and *Tsp*509I. Samples were then grouped according to RFLP profiles and compared to the predicted profiles.

Sequencing. The positive control strains for sequencing were M41, Ark-99, and Conn-A5968 (ATCC). From each amplicon, an 80-μL aliquot was submitted to 1.5% agarose gel electrophoresis and purification was performed with Wizard® SV gel and PCR clean-up system (Promega). After purification, the concentration of samples was estimated by agarose gel electrophoresis. The sequencing was conducted in a DNA automated sequencer (Applied Biosystems model 3700) at Embrapa (Cenargen, DF, Brasília) with ABI Prism® big dye terminator cycle sequencing ready reaction kit version 3.1 (PE Applied Biosystems Inc., Foster City, CA). Sequencing was performed on at least two amplicons of each strain or isolate, and at least three sequences were generated for each virus sample. Data were edited and observed with Chromas version 2.0 beta (Technelysium Pty. Ltd.). The consensus

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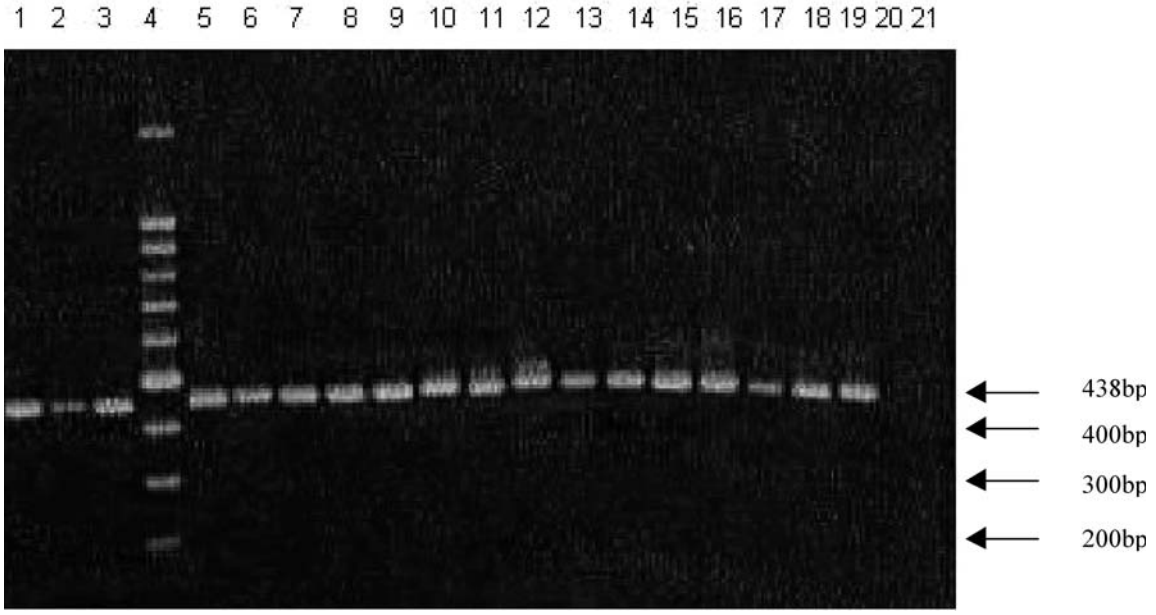

Fig. 1. Agarose gel electrophoresis of RT-PCR products (part of N gene) in 1% agarose stained by ethidium bromide. Lane 1, M41; lane 2, Conn-A5968; lane 3, Ark-99; lane 4, Molecular weight marker: 100-bp DNA ladder (Promega); lane 5, 208; lane 6, 29-78; lane 7, 297; lane 8, 283; lane 9, 290; lane 10, 327; lane 11, 351; lane 12, 319; lane 13, PM1; lane 14, PM2; lane 15, PM3; lane 16, PM4; lane 17, G; lane 18, TII; lane 19, 200; lane 20, NDV (La Sota); lane 21, Negative control (AF).

sequence was obtained from triplicate electrophoresis data of every isolate or strain.

Accession numbers. The GenBank nucleotide sequence accession numbers for the comparisons of obtained and published sequences 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; V5/90, U52595.1; N1/62, U52596.1; N9/74, U52597.1; N2/75, U52598.1; Q3/88, U52600.1; severe acute respiratory syndrome (SARS), AY307165.

RFLP analysis. The RFLP results were analyzed using the Treecon version 1.3b software for Windows (40).

Phylogenetic analysis. Translation frames were obtained by Sequence Utilities—6 Frame Translation software (BCM Search Launcher). Dendrograms were built by the neighbor-joining method with TREECON (40), and, to test the confidence of grouping, data were subject to bootstrap test ($n = 1000$). SARS (AY307165) was used as an outgroup to root the dendrogram.

RESULTS

RT-PCR. Amplified segments of 438 bp were observed for all the strains, and isolates and absent for the negative controls (Fig. 1).

RFLP, analysis, and dendrograms. The RFLP of the 438-bp amplicons resulted in the formation of three RFLP patterns for the 15 Brazilian isolates (Fig. 2). Nine Brazilian isolates (200, 208, 319, 327, 351, PM3, PM4, G, TII) showed restriction bands (approximately 240, 140, and 60 bp) similar to obtained fragments from restriction maps of the same N gene region of M41. The 140- and 60-bp bands are not shown in Fig. 2, but were weakly seen in the original photograph. Six Brazilian isolates (297, 283, 290, 29-78, PM1, and PM2) showed restriction profiles distinct of strains M41, with bands of approximately 400 and 40 bp (29-78, PM1, and PM2) and of 170 bp (297, 283, and 290). The isolates 297, 283, and 290 also showed restriction fragments distinct of all the other Brazilian isolates. The analysis of the sequencing data with WebCutter 2.0 revealed that the Brazilian isolates formed six groups (Fig. 3). Four of the Brazilian isolates (283, 290, 297, and G) were allocated together with M41, Conn-A5968, and Ark-99 (group A), although in a different branch. The Brazilian isolates obtained from clinical IB of years 1983 (283, 290, and 297; 319 and 327) or 1987 (PM1 and PM2) were grouped according to region and time period (Fig. 3). All other isolates were not grouped according to geographic area and time. Out of the nephropathogenic isolates, two were grouped together (319 and 327), but the other two (283 and 351) were grouped with the respiratory isolates (Fig. 3).

Predicted amino acid sequence and phylogenetic analysis. The dendrogram based on the predicted amino acid sequences of part of the N protein (116 amino acids) showed that all (15 of 15) of the isolates exhibited type I pattern, totally distinct of the IBV sequences available in the GenBank. A similar grouping pattern was found for Brazilian isolates as compared to the Australian isolates N9/74, N1/62, V5/90, and N2/75 (Group IV, Fig. 4).

Similarities were found in the predicted amino acid sequences among Brazilian isolates (Table 2), varying from 45% (between isolates G and 208) to 99% (PM1 and PM2) and, as compared to reference strains, varying from 42% (Q3/88 and G) to 100% (D41 and PM1). Considering the alignment of the predicted amino acid composition, a greater prevalence of basic residues was noted (data not shown). Out of the 116 analyzed amino acid residues, 18 (15%, 38%) to 24 (20%, 51%) were basic and five to nine were acid. Except for isolates 29-78 and G, 89% to 94% of basic residues were position conserved.

DISCUSSION

Fifteen Brazilian isolates were initially analyzed by RFLP. According to the RFLP analysis, the isolates were classified into six

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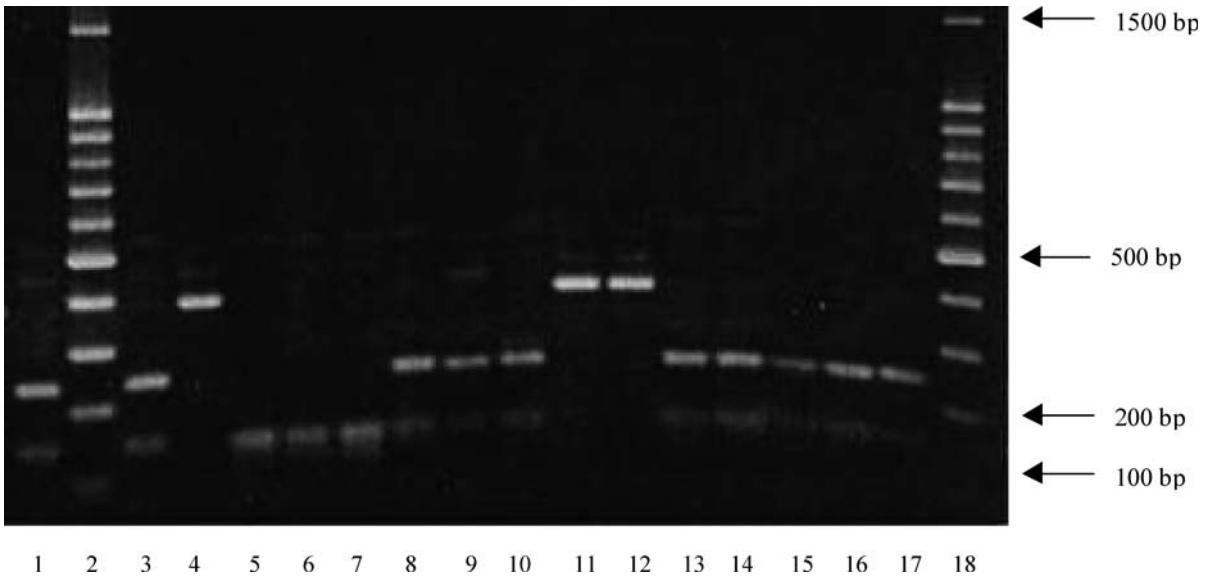

Fig. 2. RFLP patterns of the N gene of 15 IBV Brazilian isolates and reference strain (M41), digested with *Ava*II. Lanes 02 and 18: Molecular weight marker: 100-bp DNA ladder; lane 1, M41; lane 03, 208; lane 04, 29-78; lane 05, 297; lane 06, 283; lane 07, 290; lane 08, 327; lane 09, 351; lane 10, 319; lane 11, PM1; lane 12, PM2; lane 13, PM3; lane 14, PM4; lane 15, G; lane 16, TII; lane 17, 200.

genotypes. Isolates G and TII (1975); 283, 290, and 297 (1983); 327 and 351 (1983 and 1984, respectively); PM1 and PM2 (1987); and PM3 and PM4 (1989) showed a similar *Ava*II RE pattern and could be grouped according to isolation year but not according to the clinical disease, probably due to N protein not being associated to pathogenicity (12). The same grouping according to isolation year was seen for isolates 283, 290, and 297 (1983); 319 and 327 (1983); and PM1 and PM2 (1987) using *Ava*II, *Hph*I, *Sau*96I, *Tsp*509I. The isolates 283, 290, and 297; PM1 and PM2 were grouped together irrespective of the employed analyses (RFLP or sequencing), suggesting similarity. RFLP did not distinguish these isolates from the M41 reference strain, nor the G isolate from Conn-A5968 and Ark-99. However, the analysis of the predicted amino acid sequence did enable that discrimination. According to the analysis of the predicted amino acid sequence, a dendrogram was constructed and five groups were established. Group I was formed only by the Brazilian isolates, independently of the pathogenicity of the isolate, results which indicate that the Brazilian isolates do form an independent group and distinct of all IBV reported elsewhere (17,22,26,27,32,33). The isolates 283, 319, and 351 were obtained from natural IB in broilers with clinical nephritis and are closely grouped, as based on the predicted amino acids analysis. Although

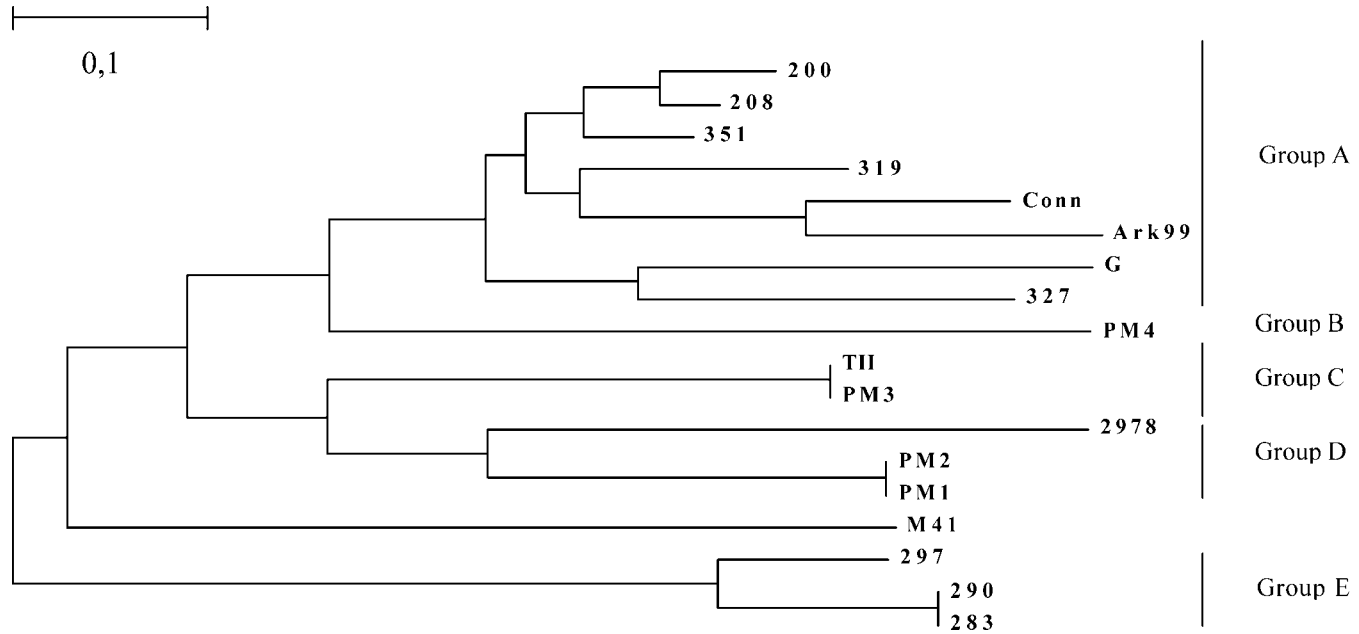

Fig. 3. Dendrogram without root of 15 Brazilian IBV isolates, M41, Conn-A5968, and Ark-99, built with TREECON. The branch length was calculated from RFLP analysis of part of the N gene with the restriction enzymes *Ava*II, *Hph*I, *Sau*96I, and *Tsp*509I. The scale represents 0.1 divergence.

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Fig. 4. Dendrogram of 15 Brazilian IBV isolates, 18 IBV and SARS (out group) available in the GenBank and EMBL, as based on the predicted amino acid sequences (116 amino acids) of part of the N protein. The dendrogram was made using the neighbor-joining method with Treecon. The dendrogram is rooted to the sequence of SARS virus (accession number AY307165). Group I, PM2, PM1, 200, PM3, TII, 351, 319, 283, 290, 297, 327, 2978, G, PM4, 208 (Brazilian IBV isolates); group II (*), Q3/88 (Australian IBV isolate); group III, Vic S (Australian IBV vaccine strain), M42, M41, H120, H52 (Massachusetts), D41, D1466 (Dutch IBV strains), ArkDPI, Ark-99, Conn, Gray, DE072, CUT2 (American North IBV strains); group IV, N9/74, N1/62, V5/90, N2/75 (Australian IBV strains), group V (**), SARS. The scale represents 0.1 divergence.

isolates allocated in group I, which caused renal disease, also caused respiratory disease, strictly respiratory isolates were observed. Several studies have demonstrated that the isolation of IBV is different and dependent on the organ sampled. The early disease is more centered in the respiratory system, from where the virus spreads to other tissues like oviduct, kidneys, intestines, and lymphoid (5,6,24). Isolates of respiratory or female reproductive system IBV were obtained from the trachea, lungs, or air sacs. Outbreaks of diarrhea and high mortality characterized by renal lesions provided the nephropathogenic isolates obtained from kidneys. The possibility exists that within the genic groups of respiratory disease nephropathogenic isolates were present, but this has not yet been experimentally verified.

In the dendrogram constructed from the predicted amino acid sequence data, a clear separation of Australian (Q3/88, V5/90, N9/74, N1/62, and N2/75) and European and U.S. isolates could be seen. Among the U.S. and European isolates, two nephropathogenic strains were observed (H52 and Gray), interspersed among the respiratory strains, suggesting the differences in N being due to different geographic origin instead of date or pathogenicity. However, it was noted that the 15 Brazilian isolates were grouped separately from Australian and the U.S. isolates, possibly indicating these being strictly Brazilian.

Despite the fact that the N protein is considered highly conserved, with a similarity greater than 80% among IBV strains (22,37,42,43,47), the data obtained show a variability of up to 55% among the Brazilian isolates and up to 58% as far as reference strains are concerned, in partial agreement with previous findings (33), which indicated 60%–63.3% of amino acid sequence identity between Australian isolates. Other authors (42) demonstrated the extremities of the N gene being less conserved.

Analyses based on the N3F10 N-specific monoclonal antibody (Mab) were previously reported (38) on the same Brazilian isolates and did not detect the PM1, PM2, PM3, 351, and 29-78 isolates, although the N3F10 antibody was not characterized according to epitope. Ignjatovic and McWatters (23) used N-specific Mab for analyzing IBV strains by enzyme-linked immunosorbent assay and Western blotting and did not detect all isolates, indicating N-based variation.

A tendency of regional IBV serotypes is reported (17,30,32, 43,45), although the occurrence of several serotypes of IBV in larger countries is described.

All (15 of 15) the Brazilian IBV isolates are distinct from the previously studied IBV in Europe, United States, and Australia, including the commercial vaccine strains. In addition, the Brazilian isolates presented large genetic diversity, independently of the date of isolation, prior to and after the official adoption of vaccination in Brazil (1980), indicating the exclusive geographic and natural evolution of IBV, reinforcing the need for evaluation of local isolates to be developed as vaccine strains.

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Table 2. Nucleotide and predicted amino acid identity (Clustal W, EMBL software) of part of the N protein gene (388 bp) of IBV reference strains (GenBank) and Brazilian isolates.

Amino acid identity (%) (116 aa)	Nucleotide identity (%)																												
	H120	M41	M42	H52	DE072	Gray	ArkDPI	Ark99	CUT2	D1466	Conn	D41	Q3/88	VicS	N1/62	TII	208	29-78	290	297	283	351	G	PM1	PM2	PM3	PM4	200	327	319
H120	—	80	80	90	81	92	93	93	93	89	94	99	60	85	83	77	79	88	77	77	76	77	77	89	89	81	81	82	77	77
M41	91	—	93	86	90	82	84	83	83	89	84	81	56	78	75	85	92	88	85	85	83	84	85	90	90	95	95	94	84	84
M42	91	93	—	83	90	82	83	82	82	89	83	80	57	78	75	85	89	88	86	86	85	85	85	90	90	92	92	93	85	85
H52	91	93	94	—	81	92	93	92	92	81	93	90	60	88	86	77	85	80	77	77	76	77	77	81	81	88	88	86	77	77
DE072	92	93	93	93	—	85	86	85	85	91	86	82	57	78	76	86	89	90	84	84	82	85	86	91	91	91	91	91	84	84
Gray	91	92	93	92	95	—	97	96	95	83	97	92	60	87	85	78	81	82	78	77	76	78	78	83	83	83	83	84	78	78
ArkDPI	93	94	95	95	96	95	—	97	97	84	99	94	60	87	86	80	82	83	79	78	77	79	79	84	84	85	85	85	79	79
Ark99	93	93	93	93	96	94	97	—	96	84	98	93	61	87	85	80	82	83	78	78	76	79	79	84	84	84	84	84	78	78
CU-T2	91	92	92	92	93	93	95	94	—	84	97	93	61	87	86	79	81	83	78	78	77	79	79	84	84	84	84	84	79	78
D1466	98	91	91	91	92	91	93	93	91	—	84	89	57	77	74	85	88	97	85	84	83	85	85	98	98	90	90	92	85	85
Conn	94	95	95	95	97	96	99	98	96	94	—	94	61	87	86	80	82	84	79	78	77	79	79	84	85	85	85	85	79	79
D41	99	92	92	92	93	92	94	93	92	99	95	—	61	85	84	78	79	88	77	77	76	77	77	89	89	81	81	83	78	78
Q3/88	62	60	63	61	61	61	62	62	62	62	62	63	—	61	61	57	55	56	57	57	56	56	57	57	57	57	57	58	57	57
VicS	85	85	90	88	87	87	87	86	84	86	87	86	62	—	87	77	76	76	78	77	76	77	77	77	77	79	79	79	76	76
N1/62	84	84	86	87	85	85	86	84	85	84	86	85	62	85	—	75	73	73	74	73	72	75	75	74	75	76	76	76	73	72
TII	87	86	87	86	86	86	87	87	86	88	88	88	62	82	82	—	83	85	90	90	88	98	98	86	86	85	85	86	90	90
208	74	75	76	78	75	75	77	76	75	75	78	75	50	69	67	74	—	88	82	82	80	83	82	89	88	96	96	93	81	82
29-78	75	68	69	70	69	69	72	70	69	75	72	75	53	67	68	68	55	—	84	83	82	84	84	98	97	90	90	91	84	84
290	84	82	86	83	82	82	85	83	82	85	85	85	62	83	80	87	68	67	—	99	98	89	89	85	85	85	85	86	94	94
297	83	81	83	82	81	81	84	82	81	84	84	84	62	82	78	87	67	66	93	—	97	89	89	85	85	84	84	86	94	94
283	77	78	79	76	75	75	78	76	76	78	78	78	57	76	73	81	66	60	98	92	—	87	88	83	84	83	82	85	92	92
351	87	86	87	86	86	86	87	87	86	88	88	88	62	82	82	100	74	68	87	87	81	—	99	86	85	85	85	86	89	90
G	55	52	55	55	52	53	55	53	54	56	55	56	42	53	56	61	45	70	58	56	51	61	—	85	85	85	85	86	89	89
PM1	96	89	89	89	90	89	92	91	89	96	93	97	62	83	82	90	77	75	85	84	78	90	56	—	99	91	92	92	85	86
PM2	95	88	89	89	89	88	92	90	88	95	92	96	62	83	82	89	76	75	85	84	78	89	56	99	—	91	91	92	85	85
PM3	90	93	92	95	92	91	93	93	91	90	94	91	60	84	84	89	82	70	83	82	76	89	55	93	93	—	99	96	84	85
PM4	78	80	81	82	80	79	81	81	80	79	82	79	54	74	71	78	94	59	72	71	67	78	50	81	81	87	—	96	84	85
200	92	91	92	93	92	91	93	93	91	92	94	93	62	84	82	90	81	72	85	84	78	90	56	95	94	98	86	—	86	86
327	68	64	67	66	65	66	68	66	66	68	68	68	51	67	67	72	51	87	69	68	62	72	75	68	68	66	56	68	—	98
319	85	82	86	84	83	83	86	84	83	86	86	86	63	85	80	90	68	68	93	93	87	90	58	86	86	84	73	86	74	—

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