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Molecular Epidemiology of Avian Infectious Bronchitis in Brazil from 2007 to 2008 in Breeders, Broilers, and Layers

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SUMMARY. Multiple lineages of Brazilian strains from 2007 to 2008 of avian infectious bronchitis virus (IBV) were detected in flocks of breeders, broilers, and layers. Organs samples from 20 IBV-positive flocks with variable clinical signs were submitted to the partial amplification of S gene (nucleotides 726-1071) of IBV. Fifteen of the 20 sequenced strains segregated in a unique Brazilian cluster subdivided in three subclusters (Brazil 01, 02, and 03). Whereas three strains could be classified as Massachusetts (Mass) genotype, the remaining two strains, originating from flocks with reproductive and respiratory disorders, grouped within the 4/91-793B genotype, a genotype that has not been detected before in Brazil. The potential relevance of the findings to the poultry industry is discussed because the low level of identity of the sequenced part of the S gene from 17 of 20 detected field strains and the vaccines of the Massachusetts serotype used suggest that the level of cross-protection by the Massachusetts vaccines might be low.

RESUMEN. Epidemiología molecular de los virus de bronquitis aviar del Brasil en el periodo entre los años 2007 y 2008 en reproductoras, pollos de engorde y gallinas de postura.

Se detectaron diferentes linajes de cepas brasileras del virus de la bronquitis infecciosa aviar en reproductoras, pollo de engorda y en gallinas de postura en el periodo comprendido entre los años 2007 y 2008. Muestras de órganos pertenecientes a 20 parvadas positivas para el virus de la bronquitis fueron sometidas a la amplificación parcial del gen S del virus de la bronquitis infecciosa (nucleótidos 726 al 1071). Quince de las 20 cepas secuenciadas se agruparon en un tronco único de cepas brasileras, subdividido en tres subgrupos (Brasil 01, 02 y 03). Mientras que tres muestras fueron clasificadas como pertenecientes al genotipo Massachusetts, las dos muestras restantes, que se obtuvieron de parvadas con problemas reproductivos y respiratorios se agruparon con el genotipo 4/91 – 793B, que es un genotipo que hasta ahora no se había detectado en Brasil. Se discute la potencial relevancia de estos resultados para la industria avícola, porque el bajo nivel de identidad en la parte secuenciada del gene S observado en 17 de las 20 muestras de campo en comparación con las vacunas Massachusetts que son utilizadas, sugiere que el nivel de protección cruzada conferida por el serotipo Massachusetts puede ser bajo.

Key words: infectious bronchitis virus, pathotypes, genotypes, 4/91, Brazil

Abbreviations: DEPC = diethyl pyrocarbonate; IB = infectious bronchitis; IBV = infectious bronchitis virus; Mass = Massachusetts; N = nucleocapsid; RT = reverse transcription; S = spike; UTR = untranslated region

Infectious bronchitis (IB) is a ubiquitous, highly complex infectious disease of poultry that is caused by multiple serotypes of avian infectious bronchitis virus (IBV). IBV is a group 3 coronavirus (order *Nidovirales*, family *Coronaviridae*) and a highly pleomorphic enveloped virus with the three envelope proteins spike glycoprotein (S), envelope, and membrane. The nucleocapsid (N) protein binds to the genomic single-stranded, positive-sense 27-kb RNA. The genome is organized as a 3′-nested set of genes, transcribed as subgenomic mRNAs by the RNA-dependent RNA polymerase coded by the open reading frame 1 that occupies the 5′ two thirds of the genome (21).

The S protein, with a size of 180 kDa, is proteolytically cleavable in two subunits S1 and S2, of 90 kDa each. S1 is the amino-terminal portion that forms the bulbs of the spike and is directly involved in receptor binding and immune response, determining the serotype of a given strain (6), whereas S2 is the carboxy-terminal subunit with a role in membrane fusion (21).

A wide range of clinical disorders in breeders, layers, and broilers can be caused by IBV infections, including respiratory disease, nephritis, reproductive failures in males and females, and enteric disease (8). A major issue for the control of IB is the potential low cross-protection of vaccine(s) used against field strains of distantly related genotypes or serotypes (8). In Brazil, the vaccine control strategies for IB are based solely on the use of attenuated vaccines of the Massachusetts (Mass) serotype. Despite the wide use of vaccination, the disease still occurs at high frequency. In these clinical outbreaks of IBV, usually a high percentage of strains of the non-Massachusetts genotype (1,23,29,30), serotype (11,13), or protectotype (11,13) has been detected. Unfortunately, the results of the IBV typing of the detected strains in these studies can hardly be combined due to the use of different techniques, genes, and/or genomic regions within these genes that were used for typing of the strains.

The aim of this survey was to assess more information about the development of the molecular diversity of IBV (based on partial S1 nucleotide sequences) in the period 2007–08 among Brazilian breeders, layers, and broilers. This knowledge is needed for the selection of strains for pathogenicity and vaccination-challenge studies to determine the potential need of IB vaccines of non-Massachusetts type for the Brazilian poultry industry.

MATERIALS AND METHODS

Source of viruses. In the period of 2007–08, a total of 20 breeders, layers and broilers flocks was included in this study (Table 1). The flocks showed disorders of the enteric, respiratory, reproductive and/or renal

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Table 1. Avian IBV field strains from Brazilian poultry included in the analysis of partial spike gene regarding bird type (broilers, layers, and breeders), age (in weeks), IBV genotype, geographic region of origin (S = southern; CW = central western; SE = southeastern; NE = northeastern), signs present at the flock at the time of sample collection, and sample in which the strains were detected.

Strain	GenBank accession	Bird type	Age (wk)	IBV type	Region	Signs	Sample
IBV/BRAZIL/2008/USP-13	FJ791254	Broilers	6	Mass	S	Res[A]	Lungs
IBV/BRAZIL/2007/USP-14	FJ791255	Layers	28	Mass	S	Ent[B], Rep[C]	Female reproductive tract
IBV/BRAZIL/2008/USP-15	FJ791256	NA[A]	NA[D]	Mass	NA	NA	Pool of organs
IBV/BRAZIL/2007/USP-16	FJ791257	Broilers	5	Brazil 03	CW	Ent	Enteric content
IBV/BRAZIL/2007/USP-17	FJ791258	Layers	27	Brazil 03	S	Ent, Rep	Female reproductive tract
IBV/BRAZIL/2007/USP-18	FJ791259	Broilers	NA	Brazil 03	S	Ren[E]	Kidney
IBV/BRAZIL/2008/USP-19	FJ791260	Grandparents	NA	Brazil	S	Res, Rep	Lung and trachea
IBV/BRAZIL/2008/USP-20	FJ791261	Broilers	5	Brazil	S	Ent	Enteric content
IBV/BRAZIL/2008/USP-21	FJ791262	Broilers	5	Brazil 02	NA	NA	Enteric content
IBV/BRAZIL/2008/USP-22	FJ791263	Broilers	3	Brazil 02	NA	NA	Enteric content
IBV/BRAZIL/2008/USP-23	FJ791264	NA	NA	Brazil 02	NA	NA	Trachea
IBV/BRAZIL/2008/USP-24	FJ791265	Broilers	7	Brazil 02	SE	Ent	Enteric content
IBV/BRAZIL/2008/USP-25	FJ791266	Layers	23	Brazil	NE	Res, Rep	Female reproductive tract
IBV/BRAZIL/2007/USP-26	FJ791267	Broilers	6	Brazil	NE	Res	Trachea
IBV/BRAZIL/2008/USP-27	FJ791268	Broilers	5	Brazil 01	S	Ent	Enteric content
IBV/BRAZIL/2008/USP-28	FJ791269	Broilers	5	Brazil 01	S	Res	Lungs
IBV/BRAZIL/2008/USP-29	FJ791270	Broilers	6	Brazil 01	S	Res	Trachea
IBV/BRAZIL/2007/USP-30	FJ791271	Breeders	45	Brazil 01	SE	Rep	Female reproductive tract
IBV/BRAZIL/2008/USP-31	FJ791272	Layers	29	4/91 (793B)	SE	Rep, Res	Trachea
IBV/BRAZIL/2007/USP-32	FJ791273	Breeders	36	4/91 (793B)	SE	Rep	Enteric content

[A]Res = respiratory.
[B]Ent = enteric.
[C]Rep = reproductive.
[D]NA = not available.
[E]Ren = renal.

tracts. Samples of lungs, tracheas, kidneys, reproductive organs, and complete enteric contents were collected from each flock as organ-specific pools (three to five birds/pool) and were sent frozen to the laboratory. The flocks were from the southern, southeastern, northeastern and central western regions of Brazil. These regions represent the major Brazilian poultry regions, with high avian population densities and flocks/farm clustering and frequencies of IBV-positive flocks ranging from 50 to 100% (4). All birds had been vaccinated against IBV using attenuated Massachusetts vaccines (broilers) or attenuated Massachusetts vaccines plus inactivated Massachusetts vaccines (layers and breeders). Also included in the study were two Massachusetts vaccines from different manufacturers continuously used in the sampled flocks, coded Mass Vaccine 01 and Mass Vaccine 02.

IBV screening and partial amplification of the spike gene. Pools were prepared as 50% (v/v) suspensions in diethyl pyrocarbonate (DEPC)-treated water and submitted to three freeze-thaw cycles in liquid nitrogen and 56 C and clarified at 5000 × *g* for 15 min at 4 C. Total RNA was extracted from the supernatants with TRIzol™ reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Each pool was surveyed for the presence of IBV by a reverse transcription (RT) seminested PCR by using primers targeted to a region of the 3′-untranslated region (UTR), highly conserved amongst IBV genotypes, as described by Cavanagh *et al.* (9).

Partial reverse transcription and amplification of the spike gene of IBV were carried out as described by Worthington *et al.* (31), resulting in amplicons of 390 bp between nucleotides 705 and 1094 (amino acids 236–364) of the S1 coding region (for strain UK/7/93, GenBank accession Z83979). For this S1 RT-PCR, one pool per flock was selected amongst those positive for the 3′-UTR RT-PCR, preferably the pool most directly related to the clinical signs observed in a given flock (Table 1). All reverse transcription and amplification steps were carried out with M-MLV Reverse Transcriptase™ and Platinum™ *Taq* DNA Polymerase (Invitrogen), respectively, according to the manufacturer's instructions.

Each test run included DEPC-treated water as the negative control and Mass vaccines as the positive controls. Exclusive rooms with restricted equipment were used for sample preparation and RNA extraction, reverse transcription, and first-step amplifications and second-round amplifications followed by electrophoresis, respectively, to avoid amplicon carryover.

Partial S gene analysis. The S1 390-bp amplicons were purified from agarose gels using the GFX™ kit (GE Healthcare, Fairfield, CT) and submitted to bidirectional DNA sequencing with Big Dye™ 3.1 (Applied Biosystems, Foster City, CA) in an ABI-377 automatic sequencer (Applied Biosystems). Sequences with Phil's Read Editor scores higher than 20 (14) were assembled with Cap-Contig application and aligned with CLUSTALW included in Bioedit 7.0.9.0 software (17), with homologous sequences retrieved from GenBank (see accession numbers in Fig. 1), and primer sequences were trimmed. A genealogic tree for the putative amino acids sequences (positions 243–357 of strain UK/7/93, GenBank accession Z83979) was built with the neighbor-joining distance algorithm and the Poisson correction, with 1000 bootstrap replicates using MEGA 4 (28). The criteria for the establishment of a cluster or lineage different from the archetypical IBV types were bootstrap value >50 and the clustering of at least three sequences.

Accession numbers. Partial S1 sequences of the 20 field strains of IBV have been assigned GenBank accessions FJ791254 to FJ791273 (Table 1), whereas Mass Vaccine 01 and Mass Vaccine 02 received the accessions FJ791274 and FJ791275, respectively.

RESULTS

IBV screening and partial amplification of the S gene. The 20 flocks included in this study showed at least one organ pool positive for the RT-PCR to the 3′-UTR of IBV. Of 19 flocks, the S1-RT-PCR was performed on the pool of organs that was most related to the clinical sign that was observed in those flocks. Of one flock

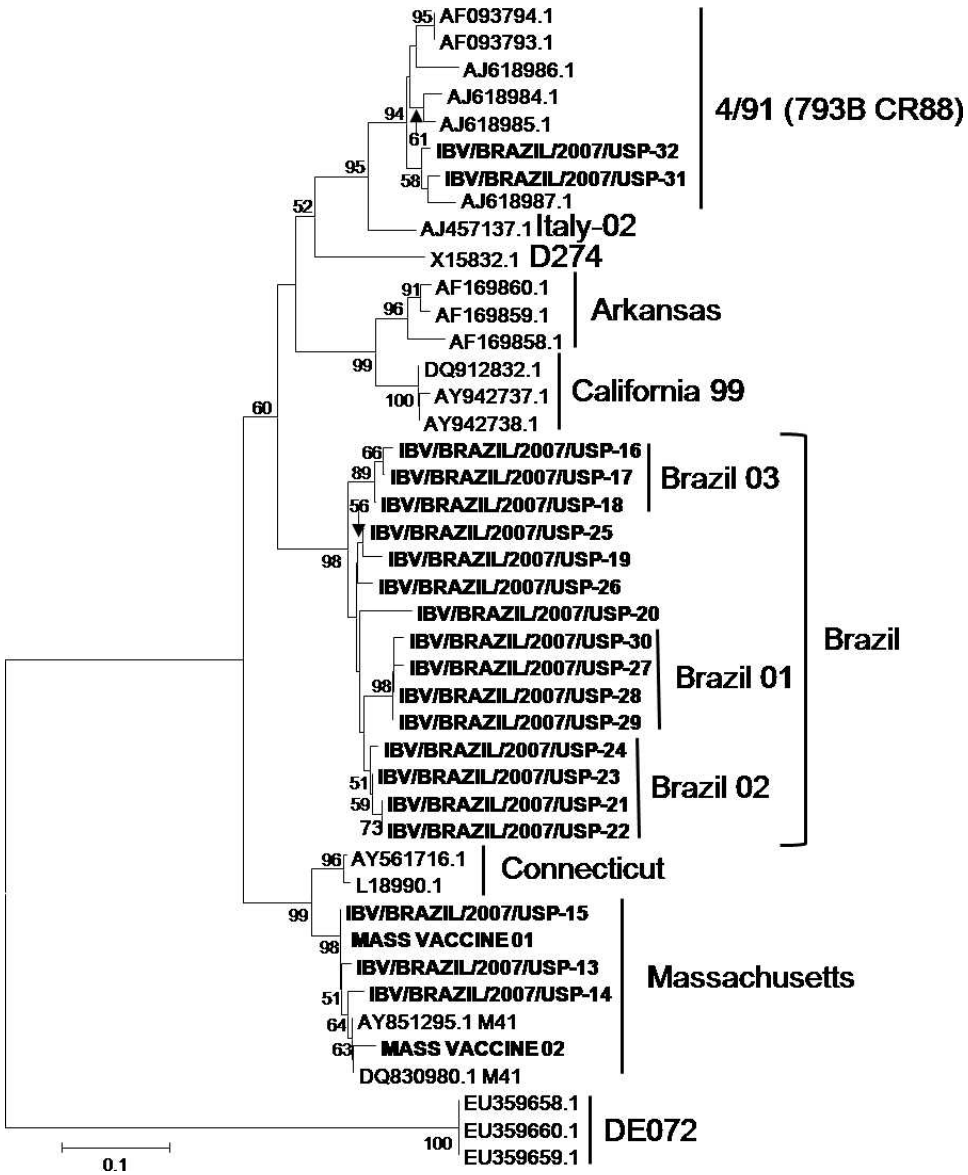

Fig. 1. Neighbor-joining distance tree with the Poisson correction for amino acids 243–357 (for strain UK/7/93, GenBank accession Z83979) of the spike glycoprotein of avian IBV of archetypical genotypes and the 20 field strains from the present study (in bold). Mass Vaccine 01 and Mass Vaccine 02 are also included in the tree. Nodes at each node are 1000 replicates bootstrap values (only those >50 are shown). The bar represents the number of amino acids substitutions per site.

(breeder flock related to strain IBV/BRAZIL/2007/USP-32) that showed reproductive disorders by the time of sample collection (Table 1), the pool of enteric contents was used for the S1-RT-PCR (instead of the pool of oviducts which was negative for IBV).

Genealogic analysis. Based on the partial S1 sequences, the 20 IBV strains were clustered in three major groups named Brazil, Massachusetts, and 4/91-793B (Fig. 1). Most strains (15) segregated in a unique Brazilian cluster. In this major cluster, named Brazil, three subclusters could be depicted (Fig. 1), named Brazil 01 (four strains), Brazil 02 (four strains), and Brazil 03 (three strains). The remaining four strains were located between subclusters Brazil 01 and 03 and did not form a specific subcluster. Regarding the three Brazilian subclusters, the most distantly related were Brazil 01 and 03, with an average amino acid identity of 91.6% (Table 2).

Concerning the archetypical genotypes included in the analysis, the highest amino acid identities for Brazil 01, 02, and 03 were related to the genotypes Connecticut (79.4%), D274 (81.1%), and 4/91 (80.5%), respectively (Table 2).

Three strains (IBV/BRAZIL/2008/USP-13, IBV/BRAZIL/2007/USP-14, and IBV/BRAZIL/2008/USP-15) detected in broilers and layers with respiratory, reproductive, and enteric disorders were classified as Massachusetts strains. One of these strains (IBV/BRAZIL/2008/USP-15) had a 100% amino acid identity with Mass Vaccine 01 and 97.39% with Mass Vaccine 02, whereas for the other two strains (IBV/BRAZIL/2008/USP-13 and IBV/BRAZIL/2008/USP-14), the amino acids identity with Mass Vaccine 01 and Mass Vaccine 02 were 99.1/96.4% and 98.2/96.4%, respectively.

Unexpectedly, two strains (IBV/BRAZIL/2008/USP-31 and IBV/BRAZIL/2007/USP-32) were classified as belonging to the genotype 4/91 (793B or CR88), previously unknown amongst Brazilian poultry. The strains were isolated from a layer flock with respiratory and reproductive disorders and from a breeder flock with

Table 2. Amino acid identities (in percent) and respective standard deviations (in parentheses) of partial spike gene sequences (positions 243–357 of strain UK/7/93, GenBank accession Z83979) amongst three clusters of Brazilian field strains of avian IBV and archetypical genotypes.

Cluster	Brazil 01	Brazil 02	Brazil 03	Brazilian 4/91	Massachusetts	D274	Arkansas	California	DE072	Connecticut	4/91
Brazil 01	99.1 (0.6)	95.6 (0.6)	91.6 (0.9)	75.4 (0.7)	79.3 (1.1)	78.9 (0.7)	77.3 (0.6)	79.1 (0.6)	41.9 (0.4)	79.4 (0.5)	75.5 (0.9)
Brazil 02		99 (0.7)	94.9 (0.9)	78 (0.7)	79.2 (1.0)	81.1 (0.5)	80.6 (0.6)	80.8 (0.6)	43.6 (0.4)	79.4 (0.5)	78.4 (1.0)
Brazil 03			98.8 (0.5)	79.7 (0.7)	77.3 (1.0)	79.2 (0.5)	79.7 (0.6)	79.8 (0.6)	43.7 (0.4)	78.3 (0.5)	80.5 (1.1)
Brazilian 4/91				98.2 (0)	73.1 (0.1)	82.4 (0)	77.6 (0.9)	77.3 (0.6)	43.2 (0)	75.4 (0.7)	94.6 (2.2)

reproductive problems that were located 963 km apart in two different states. The farms had no relation between with each other. The amino acid identity between these two strains was 98.2%. The identities of IBV/BRAZIL/2008/USP-31 and IBV/BRAZIL/2007/USP-32 were 94.7% and 93.8%, respectively, to the pathogenic 4/91 strain, with GenBank accession AF093794. The identities of these strains with the Massachusetts genotype were 73% (Table 2).

DISCUSSION

This study showed a significant variation in genotypes circulating in the Brazilian poultry industry in 2007–08. The existence of a major, characteristic Brazilian IBV genotype has already been described based on partial characterization of both the S (22,30) and the N genes (1).

The existence of the three subclusters within this Brazilian cluster showed that the genealogy of Brazilian strains of IBV is even more complicated than previously known. The detection of two strains of the 4/91-793B genotype further illustrated this increasing complexity. It's noteworthy that these two strains have been collected from flocks presenting respiratory and reproductive disorders without chest muscle lesions, conditions that have been classically associated to this type (16). The noted clinical signs were in agreement to the wider tropism described to this genotype (2). Although already described in Europe, Asia, and Middle East (3,10,25,26,27,31,32), the 4/91-793B genotype has not been detected previously in Brazilian poultry. It is interesting to note that the detection of 793B-like strains has recently been reported previously (19) in a nonspecified Latin American country, showing the emergence of strains of this genotype into Latin America. How this genotype emerged into Brazil and possibly other countries in Latin America remains unknown. It could be due to the introduction of carriers during the importation of birds from countries where 4/91 is endemic; or as already suggested in cases of introduction of new IBV types, due to the role of migratory birds as sources of infection (5,20).

It has been shown under experimental conditions (24) and field conditions that Massachusetts vaccines alone provided a low level of protection against challenge with 4/91-793B strains. The level of protection of well applied Massachusetts vaccines against strains of each of the three subclusters of the Brazilian cluster has not been determined yet. The identity of the partial sequence of S1 between Massachusetts strains and Brazil 01, 02, and 03 is 79.3%, 79.2%, and 77.3%, respectively. This region of S1 is coding for conformational epitopes partially implicated in protection and virus neutralization (18). Although the level of cross-protection that is provided by the Massachusetts vaccines (the only live IBV vaccines allowed in Brazil) against the three Brazilian subclusters cannot be predicted by the genetic information only (12), this level of differences in the S1 protein has been shown to correlate with a low-to-moderate level of cross-protection (7,15). In fact, it is to be expected that the massive and exclusive use of vaccines of the Massachusetts protectotype do provide the opportunity for field strains of non-Massachusetts protectotype to emerge as these variants meet a lower level of flock immunity (15).

Regarding the tree strains typed as Massachusetts, a vaccine origin could be assigned to strain IBV/BRAZIL/2008/USP-15, because the amino acids identity between this strain and the strain found in one of the commercial vaccines used in Brazil also included in this study (Mass Vaccine 01) was 100%. Nonetheless, for the two remaining strains (IBV/BRAZIL/2008/USP-13 and IBV/BRAZIL/2008/USP-14), this identity was below 100%, giving rise to the possibility that these are indeed wild field Massachusetts strains instead of vaccine strains.

The results of this study underline the need of further studies on the *in vitro* and *in vivo* pathogenicity and immunologic features of the IBV strains detected during this survey. This knowledge is not only applicable for the choice of the needed kind of vaccines (only Massachusetts or more) and vaccination schedules but also to the proposition of genetic markers to the molecular epidemiology of IBV strains and their pathogenicity. These studies will be helpful to minimize the negative impact of these IBV strains for the individual chicken and the poultry industry.

REFERENCES

1. Abreu, J. T., J. S. Resende, R. B. Flatschart, A. V. Folgueras-Flatschart, A. C. Mendes, N. R. Martins, C. B. Silva, B. M. Ferreira, and M. Resende. 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. Avian Dis. 50:494–501. 2006.

2. Adzhar, A., R. E. Gough, D. Haydon, K. Shaw, P. Britton, and D. Cavanagh. Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. Avian Pathol. 26:625–640. 1997.

3. Bochkov, Y. A., G. V. Batchenko, L. O. Shcherbakova, A. V. Borisov, and V. V. Drygin. Molecular epizootiology of avian infectious bronchitis in Russia. Avian Pathol. 35:379–393. 2006.

4. Brandão, P. E., T. L. Sandri, S. P. Souza, S. L. Kuana, L. J. Richtzenhain, and L. Y. B. Villarreal. Recombination, point mutations and positive selection on the basis of the molecular diversity of Brazilian strains of avian infectious bronchitis virus. In: Proc. VI International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens, Rauischholzhausen, Germany. pp. 47–58. 2009.

5. Cavanagh, D. Coronaviruses in poultry and other birds. Avian Pathol. 34:439–448. 2005.

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

7. Cavanagh, D., M. M. Elus, and J. K. Cook. Relationship between sequence variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection in vivo. Avian Pathol. 26:63–74. 1997.

8. Cavanagh, D., and J. J. Gelb. 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.

9. Cavanagh, D., K. Mawditt, D. de B. Welchman, P. Britton, and R. E. Gough. Coronaviruses from pheasants (*Phasianus colchicus*) are genetically closely related to coronaviruses of domestic fowl (infectious bronchitis virus) and turkeys. Avian Pathol. 31:81–93. 2002.

10. Cook, J. K., S. J. Orbell, M. A. Woods, and M. B. Huggins. A survey of the presence of a new infectious bronchitis virus designated 4/91 (793B). Vet. Rec. 138:178–180. 1996.

11. Cook, J. K. A., S. J. Orbell, M. A. Woods, and M. B. Huggins. Breadth of protection of the respiratory tract provided by different live-attenuated infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotypes. Avian Pathol. 28:477–485. 1999.

12. De Wit, J. J. Detection of infectious bronchitis virus. Avian Pathol. 29:71–93. 2000.

13. Di Fabio, J. L., I. Rossini, S. J. Orbell, G. Paul, M. B. Huggins, A. Malo, B. G. Silva, and J. K. Cook. Characterization of infectious bronchitis viruses isolated from outbreaks of disease in commercial flocks in Brazil. Avian Dis. 44:582–589. 2000.

14. Ewing, B., and P. Green. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res. 8:186–194. 1998.

15. Gelb, J. Jr, Y. Weisman, B. S. Ladman, and R. Meir. S1 gene characteristics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996 to 2000). Avian Pathol. 34:194–203. 2005.

16. Gough, R. E., C. J. Randall, M. Dagless, D. J. Alexander, W. J. Cox, and Pearson, D. A 'new' strain of infectious bronchitis virus infecting domestic fowl in Great Britain. Vet. Rec. 130:493–494. 1992.

17. Hall, T. A. BioEdit: a user-friendly biological sequence alignment editor and analyses program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41:95–98. 1999.

18. Ignjatovic, J., and S. Sapats. Identification of previously unknown antigenic epitopes on the S and N proteins of avian infectious bronchitis virus. Arch. Virol. 150:1813–1831. 2005.

19. Jones, R. C., C. E. Savage, K. J. Worthington, and L. A. Hughes. Observations on global and local epidemiology of avain coronaviruses. In: Proc. VI International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens, Rauischholzhausen, Germany. pp. 2–6. 2009.

20. Liu, S., J. Chen, X. Kong, Y. Shao, Z. Han, L. Feng, X. Cai, S. Gu, and M. Liu. Isolation of avian infectious bronchitis coronavirus from domestic peafowl (*Pavo cristatus*) and teal (*Anas*). J. Gen. Virol. 86:719–725. 2005.

21. Masters, P. S. The molecular biology of coronaviruses. Adv. Virus Res. 66:193–292. 2006.

22. Montassier, M. F. S., L. Brentano, L. J. Richtzenhain, and H. J. Montassier. Genetic diversity on S1 glycoprotein of avian infectious bronchitis virus strains isolated in Brazil between 1988–2000. In: Proc. V International Symposium on Corona- and Pneumovirus Infections, Rauischholzhausen, Germany. pp. 119–131. 2006.

23. Montassier, M. F. S., L. Brentano, H. J. Montassier, and L. J. Richtzenhain. Genetic grouping of avain infectious bronchitis virus isolated in Brazil based on RT-PCR/RFLP analysis of the S1 gene. Pesqui Vet. Bras. 28:190–194. 2008.

24. Parsons, D., M. M. Ellis, D. Cavanagh, and J. K. Cook. Characterisation of an infectious bronchitis virus isolated from vaccinated broiler breeder flocks. Vet. Rec. 131:408–411. 1992.

25. Roussan, D. A., W. S. Totanji, and G. Y. Khawaldeh. Molecular subtype of infectious bronchitis virus in broiler flocks in Jordan. Poult. Sci. 87:661–664. 2008.

26. Seyfi Abad Shapouri, M. R., M. Mayahi, K. Assasi, and S. Charkhkar. A survey of the prevalence of infectious bronchitis virus type 4/91 in Iran. Acta Vet. Hung. 52:163–166. 2004.

27. Shimazaki, Y., T. Horiuchi, M. Harada, C. Tanimura, Y. Seki, Y. Kuroda, K. Yagyu, S. Nakamura, and S. Suzuki. Isolation of 4/91 type of infectious bronchitis virus as a new variant in Japan and efficacy of vaccination against 4/91 type field isolate. Avian Dis. 52:618–622. 2008.

28. Tamura, K., J. Dudley, M. Nei, and S. Kumar. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596–1599. 2007.

29. Villarreal, L. Y., P. E. Brandao, J. L. Chacon, M. S. Assayag, P. C. Maiorka, P. Raffi, A. B. Saidenberg, R. C. Jones, and A. J. Ferreira. Orchitis in roosters with reduced fertility associated with avian infectious bronchitis virus and avian metapneumovirus infections. Avian Dis. 51:900–904. 2007.

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

31. Worthington, K. J., R. J. Currie, and R. C. Jones. A reverse transcriptase-polymerase chain reaction survey of infectious bronchitis virus genotypes in western Europe from 2002 to 2006. Avian Pathol. 37:247–257. 2008.

32. Xu, C., J. Zhao, X. Hu, and G. Zhang. Isolation and identification of four infectious bronchitis virus strains in China and analyses of their S1 glycoprotein gene. Vet. Microbiol. 122:61–71. 2007.

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