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Genetic grouping of avian infectious bronchitis virus isolated in Brazil based on RT-PCR/RFLP analysis of the S1 gene[1]

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ABSTRACT.- Montassier M.F.S., Brentano L., Montassier H.J. & Richtzenhain L.J. 2008. **Genetic grouping of avian infectious bronchitis virus isolated in Brazil, based on RT-PCR/RFLP analysis of the S1 gene.** *Pesquisa Veterinária Brasileira 28(3):190-194.* Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando de Paiva 87, São Paulo, SP 05508-000, Brazil. E-mail: leonardo@usp.br

Twelve Brazilian isolates and one reference vaccine strain of avian infectious bronchitis virus (IBV) were propagated in embryonating chicken eggs. The entire S1 glycoprotein gene of these viruses was analysed by reverse-transcriptase-polymerase chain reaction and restriction fragment length polymorphism (RT-PCR-RFLP), using the restriction enzymes *Hae*III, *Xcm*I and *Bsty*I. The RFLP patterns led to the classification of these isolates into five distinct genotypes: A, B, C, D and Massachusetts. Five of twelve isolates were grouped in Massachusetts genotype and the remaining seven viruses were classified into four distinct genotypes: A (2), B (2), C (2) or D (1). Such genotyping classification agreed with previous immunological analysis for most of these viruses, highlighting the occurrence of a relevant variability among the IBV strains that are circulating in Brazilian commercial poultry flocks.

INDEX TERMS: Avian infectious bronchitis virus, spike glycoprotein, genotyping and RFLP.

RESUMO.- [**Agrupamento genético de isolados do vírus da bronquite infecciosa das aves no Brasil com base na análise do gene S1 por RT-PCR-RFLP**.] Doze isolados de campo do Brasil e uma estirpe de referência vacinal do vírus da bronquite infecciosa das aves (VBI) foram propagadas em ovos embrionados SPF. O gene S1 dessas amostras foi analisado por RT-PCR seguido de RFLP, empregando-se as enzimas de restrição *HaeIII, XcmI e BstyI.* Observou-se a existência de cinco genotipos diferentes: M (Massachusetts), A , B, C e D. Cinco dos doze isolados de campo do VBI foram classificados no genótipo Massachusetts e os sete vírus restantes foram classificados em quatro genotipos diferentes; A (2), B (2), C (2) ou D (1). Os resultados desta genotipagem concordam com os dados obtidos na análise imunológica previamente realizada para a maior parte destes vírus, destacando a ocorrência de uma variabilidade marcante entre os isolados do VBI que estão circulando nas granjas avícolas comerciais do Brasil.

TERMOS DE INDEXAÇÃO: Bronquite infecciosa das aves, glicoproteina S, genotipagem e RFLP.

INTRODUCTION

The avian infectious bronchitis virus (IBV), the prototype of the *Coronaviridae* family, is the etiologic agent of infectious bronchitis (IB), an acute highly contagious disease of the respiratory and urogenital tracts of chickens. The viral genome is a single-stranded, non-segmented RNA molecule of positive polarity. The virus has a worldwide distribution and many variants with changes in the genome have been identified. These changes include

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deletions, insertions, point mutations and, in some cases, recombinations, which occur continuously in the nature (Cavanagh & Naqi 2003).

IBV virions contain four structural proteins: spike, membrane, small membrane, and nucleocapsid proteins. The spike glycoprotein of IBV is composed by two glycopeptides: S1 (90 kD) and S2 (84 kD). The S1 glycopeptide is found at the distal end of the spike protein and contains important epitopes that induce both virus-neutralizing and hemagglutining-inhibiting antibody (Cavanagh et al.1986). S1 region varies greatly in its amino acid sequence and the variations are thought to be located within two hypervariable regions (HVRs) (Smati et al. 2002). Therefore, serotypic evolution in IBV seems to be associated primarily with the sequences of the S1 glycoprotein (Liu et al. 2006).

Classification systems of IBV strains are divided into two major groups: functional tests, which regard the biological function of a virus; and non-functional tests, which look at viral genome. Typing by functional tests, results in pathotypes, protectotypes and antigenic types (De Wit 2000, Cavanagh 2005). The pathotype is defined by tissue tropism and predominant lesions displayed by an IBV strain in natural hosts (Cavanagh 2005). Grouping of IBV strains into protectotypes provides direct data about the efficacy of a vaccine and strains that induce protection against each other belong to the same protectotype. Antigenic typing characterizes the serotypes and epitope-types of an IBV strain, using respectively, virus-specific polyclonal or monoclonal antibodies. The classification of IBV strains based on characterization of the genome results in genotypes. The main genotyping methods include nucleotide sequencing, detection of genotype-specific parts of the genome by RT-PCR, or determining the position of enzyme cleavage sites on a relevant gene by the restriction fragment length polymorphism technique (RFLP) (De Wit 2000).

The first isolation of IBV was recorded in Brazil in 1957 (Hipólito 1957) and despite the official introduction of IBV vaccination in 1979, several outbreaks of infectious bronchitis have been ongoing (Ito 2006). The majority of Brazilian IBV field isolates recovered up to 1989 were classified as Mass (Massachusetts) serotype (Ito 2006). However, Di Fabio et al. (2000) found, among fifteen field IBV isolates, only one as belonging to the Mass serotype and the other fourteen were classified into distinct antigenic groups from ones described previously in other countries.

In 1993, a new method for molecular typing of IBV was developed and combines the reverse transcriptase-polymerase chain reaction (RT-PCR) followed by restriction fragment length polymorphism (RFLP) analysis of the S1 gene. The RFLP patterns of this gene correlate with the main IBV serotypes (Kwon et al. 1993, Jackwood et al. 1997, 2001, 2005, Callison et al. 2001). The method is rapid and has led to the identification of a high number of virus isolates, which was not possible with the traditional tests (Jackwood et al. 1997, 2001, 2005). These features make the S1 glycoprotein gene of IBV an important target to be analyzed by a technique such as RFLP.

In Brazil, data on the molecular genetics of IBV field isolates have only been recently reported, and even lesser results of investigations relating antigenic types and genotypes of these viruses have become available. Therefore, the present study was focussed on the genetic characterization by RT-PCR-RFLP, of the S1 glycoprotein gene of twelve IBV strains isolated from southern and south-eastern regions in Brazil from 1988 to 2000, as well as on the relationships of the genotypes identified, to antigenic and pathological characteristics previously recorded for these viruses.

MATERIALS AND METHODS

IBV strains, isolates and virus propagation. All twelve IBV isolates used in this study were obtained from Regional Laboratories of Avian Pathology, Embrapa-CNPSA (Concórdia, SC) and SPAVE (São Paulo, SP) in Brazil. The viruses were isolated from different tissue samples collected from commercial broiler or layer flocks in southern and south-eastern regions. Seven of the poultry flocks sampled had been vaccinated with live or attenuated Mass viral strain (H120) vaccines; and three were not vaccinated; the vaccination status of the other two flocks was unknown (Table 1). The IBV isolates as well as the reference H120 IBV strain were propagated in 9-10 day old specific pathogen free (SPF) embryonated chicken eggs and identified as IBV by Con A-S-ELISA (Bronzoni et al. 2005).

RT-PCR amplification. Viral RNA was extracted from allantoic fluid of inoculated eggs using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations with the inclusion of the RNAguard™ (Amersham Biosciences). Reverse-transcriptase (RT) reaction were carried out with SuperScript II (Invitrogen), using random hexamers; the first-strand cDNA was immediately used for amplification by PCR. The RT mixture was incubated at 42°C for 60 min, and subsequently heated for 10 min at 72°C to stop the enzymatic reaction. Amplification of the whole S1 gene was carried out by PCR and using the forward S1OLOGO5': 5'TGAAACTGAACAAAAGACA3' and the reverse S1OLIGO3': 5' CATAACTAACATAAGGGCAA3' primers previously described (Kwon et al. 1993). For the PCR reaction, 20 pmols of each primer and 5mL of cDNA were added to pre-mixed (5mL of

Table 1. Infectious bronchitis virus isolates from field cases in chickens in Brazil analyzed in this study

IBV strains	Form of the disease	Year of isolation	Region	Immune status
IBVPR-01	Respiratory	1988	Southeast	Unknown
IBVPR-02	Respiratory	1988	Southeast	Unknown
IBVPR-03	Renal	2000	Southeast	Vaccinated
IBVPR-04	Respiratory	2000	Southeast	Unvaccinated
IBVPR-05	Respiratory	2000	Southeast	Unvaccinated
IBVPR-06	Respiratory	2000	Southeast	Unvaccinated
IBVPR-07	Respiratory	1999	Southeast	Vaccinated
IBVSP-01	Renal	1998	Southeast	Vaccinated
IBVSP-02	Renal	1999	Southeast	Vaccinated
IBVSC-01	Renal	1999	South	Vaccinated
IBVSC-02	Respiratory	1999	South	Vaccinated
IBVSC-03	Respiratory	1999	South	Vaccinated

10xPCR buffer, 0.2mM each dNTP, 1.5mM, Mg_2SO_4, 2.0 units of Platinum® Taq High Fidelity) and autoclaved-distilled water to complete 50mL. PCRs was performed using 35 cycles of denaturation at 94°C for 1min, annealing at 45°C for 2min and extension at 68°C for 5min. The initial denaturation and extension steps were run at 94°C for 5min. and at 74°C for 6min., respectively. Final extension was conducted at 74°C for 10min. PCR products were analyzed on a 1% agarose gel containing (0.5mg/mL) of ethidium bromide.

RFLP analysis. PCR products of the desired size (about 1720bp) were excised from the agarose gel and purified using GFX PCR DNA Gel Band Purification Kit (GE Healthcare Biosciences). The restriction enzymes *Hae III, BstYI and XcmI* (New England Biolabs) were used according to manufacturer's specifications for digestion of the PCR products of the S1 gene. These were separated by electrophoresis in 2% of agarose gels stained with ethidium bromide. The RFLP patterns of the field isolates were then compared with those of the digested vaccine reference strain PCR amplicon.

RESULTS AND DISCUSSION

Genetic and phenotypic variants of IBV are continuously evolving in commercial chicken flocks; their periodic emergence can lead to widely spread disease. These viruses are considered by RT-PCR-RFLP as variants, when a band pattern generated after digestion of the S1 glycoprotein gene with one or more restriction enzymes, and electrophoresis separation, shows differences in size or number of bands, or in both parameters (Jackwood et al. 2005). Thus molecular techniques like RFLP analysis, can be useful for the identification of field strains related to vaccines and their novel variants (Gelb et al. 2005).

In the present study, RT and PCR associated with RFLP analysis were employed to genotype twelve Brazilian IBV isolates and to compare them with the digestion patterns of the S1 gene amplified from a H120 vaccine strain of IBV. The S1 glycoprotein gene of all isolates were amplified by RT-PCR and showed to have the predicted size of approximately 1.72 kb (Fig.1).

The digestion of the amplified S1 gene with the restriction enzyme *HaeIII* generated four different RFLP patterns (Fig.2). The isolates IBVPR03, IBVSP01, IBVPR07, IBVSC01 and IBVSC03 showed a RFLP profile corresponding to the *HaeIII* digestion of S1 gene of the reference H120 strain (Mass genotype). The other seven viruses displayed three RFLP patterns, which were distinct

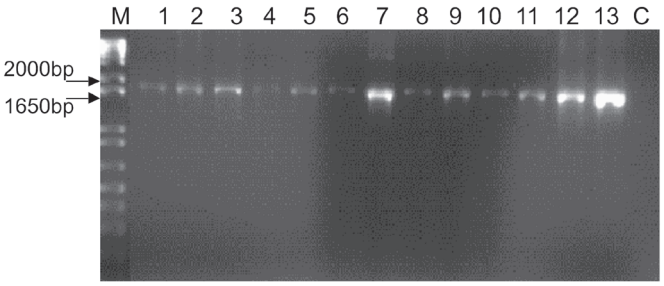

Fig.1. Eletrophoretic profile of S1 gene PCR products (@1.72 kb) produced by amplifying the reference strain and Brazilian isolates of infectious bronchitis virus (IBV) using S1-OLIGO-5' and S1-OLIGO-3 primers. M: 1kb plus DNA Leader (Invitrogen; 1-13:IBVPR01, IBVPR02, IBVPR03, IBVPR04, IBVPR05, IBVPR07,IBVSP01,IBVSP02 IBVSC02,IBVSC03, IBVSC01,IBVPR06 and H120. C: Negative control.

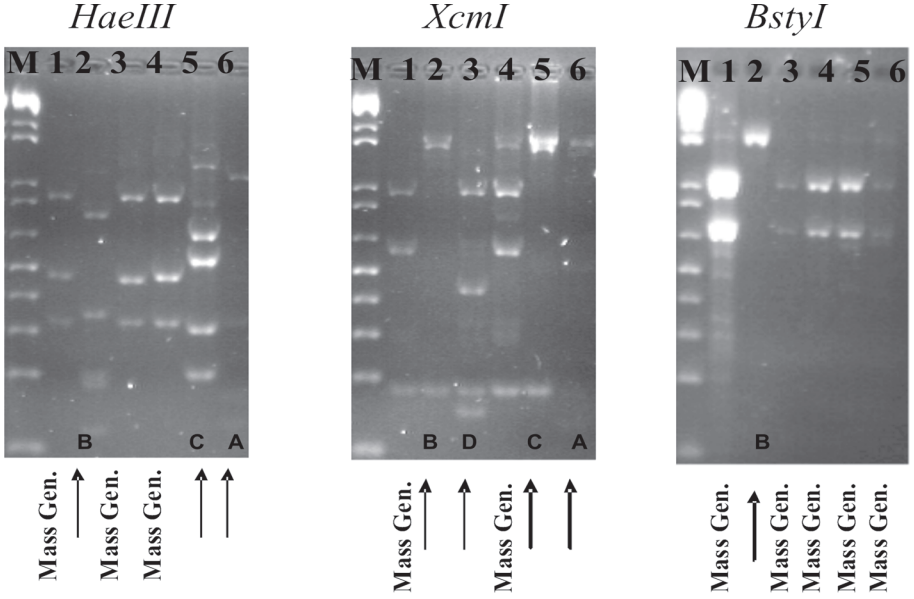

Fig.2. Illustrative electrophoretic profiles of S1 gene PCR product of five Brazilian field isolates, amplified with the oligonucleotides S1-OLIGO-5' and S1-OLIGO-3' and digested with endonucleases *HaeIII* , *XcmI* and *BstyI* . Lane M: 1kb plus DNA marker; Lane 1: H120 strain; Lane 2: IBVPR01; Lane 3: IBVSC02; Lane 4: IBVSC01; Lane 5: IBVPR06; and Lane 6: IBVSP02. Mass and four distinct genotypes (A, B, C and D, arrows) were identified.

from Mass genotype and from each other. They were classified as A, B and C genotypes containing respectively, the isolates IBVPR04 and IBVSP02 (Genotype A), the isolates IBVPR01, IBVPR02 (Genotype B), or the isolates IBVPR05 and IBVPR06 (Genotype C), (Fig.2). The digestion of the S1 gene amplicons with the enzyme *XcmI* distinguished, besides the same four profiles identified above, an additional genotype (Genotype D), which discriminated the isolate IBVSC02 from the others (Fig.2). RFLP analysis using the enzyme *BstyI*, confirmed the classification of the isolates IBVPR01 and IBVPR02 as containing Genotype B, because a singular RFLP pattern was generated by the digestion of the S1gene of these two viruses, differentiating them from the Mass genotype, as well as from patterns recorded for the remaining Brazilian IBV isolates (Fig.2).

Interestingly, all five isolates classified as of Mass Genotype, were obtained from vaccinated flocks, despite the finding at necropsy, that the affected birds had developed respiratory or renal lesions. Actually these were expected results, because the H120 attenuated vaccine has been widely adopted in Brazil after official introduction of IBV vaccines in 1979 (Ito 2006). Furthermore, the isolation of IBV viruses sharing genetic and phenotypic characteristics with vaccine reference strains, was not a solitary finding since it had also been reported as a result of virus evolution in various countries, such as United States, Sweden, Egypt and Israel (Farsang et al. 2002, Gelb et al. 2005, Jackwood et al. 2005, Abdel-Monein et al. 2006). There, isolates classified as being of the Massachusetts genotype, were revealed by molecular techniques to be closely related to the H120 strain, even though expressing different pathological activities (Farsang et al. 2002, Gelb et al. 2005, Jackwood et al. 2005, Abdel-Monein et al. 2006). In contrast, among the viruses classified in the four additional genotypes distinct from Massachusetts, there were isolates from vaccinated (IBVSP-02, and IBVSC-02), or non-vaccinated flocks (IBVPR04, IBVPR05 and IBVPR06), and also from flocks of unknown vaccination status (IBVPR01 and IBVPR02).

The RFLP genotyping here performed agreed with previous results obtained by the antigen characterization, by virus-neutralization, of some of the analyzed viruses. Thus, high virus neutralizing relationships ($\geq$0.94) were observed when comparing the vaccine strain H120 with the five viruses classified to be of Mass genotype, while low serological relationships ($\leq$0.52) to this reference strain, were found by comparing the viruses grouped in the variant genotypes (A, B, C or D) (Wentz 1992, Montassier et al. 2003, Santos et al. 2004).

In addition to the agreement of the identified RFLP patterns for the Brazilian viruses and the serotyping of some of the IBV isolates, partial results from protection tests, confirmed that the H120 vaccine was also able to induce a high protection against infection by most of the viruses classified as of Mass genotype, recorded for the isolates IBVPR03, IBVPR07 and IBVSC01 (Montassier et al. 2003, Santos et al. 2004, Pereira et al. 2006). Conversely, this vaccine strain provided low protection against the isolate IBVSC02 (Montassier et al. 2003).

Thus, the current RFLP genotyping results, as previously observed (Kwon et al. 1993, Wang & Huang 2000), were correlated with those obtained in immunological analysis. However, discrepancy between RFLP patterns and immune-typing of IBV isolates can occur, and as reported by Hein et al. (1998), different USA isolates typed by RFLP as belonging to the same genotype, can show different serotypes or protectotypes.

As opposed to the correlation between genotyping and immune-typing for IBV strains, the IBVPR07 isolate, that was classified in Mass genotype, had shown the ability to replicate and cause marked lesions in gonads without inducing significant alterations in respiratory system of experimentally infected chickens (Pereira et al. 2006). These features led to classify this virus in a different pathotype from that of Mass viruses, which typically replicate in respiratory tract (Cavanagh 2005). Interestingly, an IBV isolate from Egypt, which was classified in Mass genotype based on S1 gene nucleotide sequencing, displayed a distinct pathotype and affect more intensively the kidneys of experimentally infected birds (Abdel-Monein et al. 2006). Such divergence is not uncommon finding when the RFLP patterns and the pathogenicity of IBV isolates are compared, because the pathotypes of these viruses did not correspond to their genotypes, as based on S1 gene analysis (Wang & Huang 2000).

Antigenic, genetic, pathological and epidemiological analysis of IBV isolates in Brazil has not been performed in a systematic fashion. The genetic relationships between Brazilian isolates and vaccine or foreign IBV strains also remain barely known. However, this study demonstrated the occurrence of high variability among IBV Brazilian isolates, as some recent investigations found about the antigenic and genetic variability of nucleotide sequences occurring among some Brazilian isolates (Di Fabio et al. 2000, Abreu et al. 2006a,b, Brentano et al. 2006, Villarreal et al. 2006a,b**).**

However, studies using RFLP to characterize IBV variants, especially of the S1 gene, isolated in Brazil, are scarce and based only on nucleoprotein gene (Abreu et al. 2006a,b). That molecular technique developed in the early of 90's (Kwon et al. 1993) has been continuously applied to the analysis of IBV variants in the USA, Canada, China and Korea (Song et al. 1998, Smati et al. 2002, Gelb et al. 2005, Jackwood et al. 2005). The current results confirm the ability of RFLP analysis to screen and perform a genetic characterization of Brazilian IBV isolates and to indicate some relevant phenotypic variations related to the antigenicity of these viruses; nevertheless, further investigation is required in order to characterize changes caused by mutations and/or recombinations at the nucleotide and amino acid levels of the S1 glycoprotein of these isolates more accurately.

To conclude, the main results of the present study, do

point out the presence of significant genetic variability among Brazilian IBV field isolates, here identified by RFLP analysis of the S1gene.

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