Nephritis Associated with a S1 Variant Brazilian Isolate of Infectious Bronchitis Virus and Vaccine Protection Test in Experimentally Infected Chickens

Filipe Santos Fernando¹, Maria de Fátima da Silva Montassier¹, Ketherson Rodrigues Silva¹, Cintia Hiromi Okino¹, Elisabete Schirato de Oliveira¹, Camila Cesário Fernandes¹, Márcio de Barros Bandarra², Mariana Costa Mello Gonçalves³, Mariana Monezi Borzi¹ Romeu Moreira dos Santos¹, Rosemeri de Oliveira Vasconcelos³, Antônio Carlos Alessi¹, Hélio José Montassier¹

¹School of Agricultural and Veterinary Sciences, São Paulo State University (FCAV-Unesp), Department of Veterinary Pathology, Laboratory of Veterinary Immunology and Virology, Jaboticabal 14884-900, SP, Brazil
²Embrapa Swine and Poultry, Concórdia 89700-000, SC, Brazil
³School of Agricultural and Veterinary Sciences, São Paulo State University (FCAV-Unesp), Department of Veterinary Pathology, Laboratory of Veterinary Pathology, Jaboticabal 14884-900, SP, Brazil
⁴Federal Institute of Goiás - Rio Verde 75901-970, GO, Brazil

Abstract: Infectious bronchitis virus (IBV) induces a significant negative impact on poultry production worldwide, specially due to the continuous emergence of viral variants. This virus causes damage to the respiratory tract, and depending on the virus strain, affects and damages the urogenital system. The objective of this study is to characterize the pathotype and the cross-immunity with regard to Massachusetts vaccine strain (H120) of a Brazilian IBV field isolate (IBVPR-05) previously S1 genotyped as a variant. The pathogenicity test was conducted on two experimental groups of specific pathogen free chickens; one was vaccinated with attenuated Massachusetts H120 strain and the other remained non-vaccinated. Three weeks after vaccination, both groups were challenged with IBRPR-05. The tracheal ciliostasis, and the viral load, histopathology and immunohistochemistry in trachea and kidney samples were evaluated. The viral loads, measured by quantitative real time RT-PCR, were higher in kidney than in trachea, and the most prominent histopathological changes were found in the kidneys. The renal lesions were characterized by the presence of nephritis with intense inflammation, tubular epithelial cell degeneration and necrosis. The H120 vaccine induced a partial protection against the infection of trachea and kidney tissues by this variant isolate. Thus, the Brazilian variant isolate IBVPR-05 was characterized in this study as a nephropathogenic pathotype and as a protocotype differing from Massachusetts vaccine strain of IBV. This indicates the importance to determine these biologic characteristics of other Brazilian variant IBV isolates, in order to implement more effective control measures of IBV infection in this country.

Key words: Avian infectious bronchitis virus, variant, nephropathogenic, nephritis, Massachusetts vaccine

INTRODUCTION

The avian infectious bronchitis virus (IBV) belongs to the genus Gammacoronavirus of the Coronaviridae family and is the etiologic agent of infectious bronchitis (IB), and has been one of the major pathogens affecting the global poultry industry. IBV infects chickens of all ages and causes lesions in respiratory and urogenital organs (Cavanagh, 2007; Cook, 2002). IBV initially replicates the upper respiratory tract and may then infect other epithelial cells of other organs of the host (Dhinaikut and Jones, 1997). Some IBV strains replicate in the kidney, and due to their nephropathogenic properties, have the potential to cause severe losses (Cavanagh and Naqi, 2003; Cavanagh, 2005). In addition, the presence of IBV renal epithelial cells generates an acute interstitial inflammatory response with inflammatory cells proliferation and the development of necrosis and nephritis (Abdel-Moneim et al., 2006). It has been shown that the spike glycoprotein (S) of corona virus is a determinant of cell tropism (Kuo et al., 2000). Because of the high rates of glycoprotein S1 gene mutation, several IBV serotypes or antigenic variant strains have been reported in many countries (Cavanagh, 2007). In general, different IBV serotypes of the virus do not confer cross protection against each other (Ignjatovic and Sapats, 2000), and cross protection tends to diminish as the degree of amino acid identity

Corresponding Author: Filipe Santos Fernando, School of Agricultural and Veterinary Sciences, São Paulo State University (FCAV-Unesp), Department of Veterinary Pathology, Laboratory of Veterinary Immunology and Virology, Jaboticabal 14884-900, SP, Brazil
between the S1 proteins of IBV strains decreases (Gelb et al., 2005). However, some strains of the virus are effective in inducing cross protection against other serotypes or genotypes and these viruses are classified in the same protecotypes (Dhinakar and Jones, 1998).

In Brazil, the presence of a significant number of genetic variants of IBV with characteristics differing from reference IBV strains from other countries, have been reported with increasing frequency since the year 2000 (Montassier et al., 2008; Montassier et al., 2012; Villarreal et al., 2007; Filipe et al., 2010). However, the characteristics of these genetic variants were not associated with phenotypic variations, such as those related to cell tropism and pathogenesis. Another aspect that remained unknown is the protection conferred against these variant Brazilian IBV isolates by the attenuated live vaccine strain from Massachusetts serotype (H120 and Ma5), which is the only attenuated vaccine authorized in Brazil.

This paper investigates and characterizes the tissue tropism and pathogenicity of a Brazilian IBV isolated previously genotyped as a S1 variant (IBVPR-05 – GenBank: GQ169242) (Montassier et al., 2008). In addition, the protection induced by a commercially available IBV vaccine in Brazil was also evaluated for this variant isolate in order to characterize its protecotype with regard to the Massachusetts vaccine strain.

MATERIALS AND METHODS

**Virus:** The IBVPR-05 Brazilian field isolate used in this study was originated from a broiler flock showing respiratory clinical signs. This virus was genotyped by RFLP and nucleotide sequencing (Genbank GQ169242), after amplification of S1 gene (Montassier et al., 2008; Montassier et al., 2012). The virus was propagated in 9-to 11-day-old embryonated chicken eggs, and the embryo 50% infectious doses (EID<sub>50</sub>) were determined according to Reed and Muench (1938).

**Experimental design:** We used 18 one-day old “Specific Pathogen Free” (SPF) birds irrespective of sex. The birds were divided into three groups and maintained in isolators with positive pressure in air-conditioned rooms with negative pressure. One group (Non vac) was composed of six birds and was challenged at the sixth week of age with field strain IBVPR-05 with 50 μl containing 1 x 10<sup>10</sup> EID<sub>50</sub> by intranasal and intraocular routes. They were euthanized five days after challenge. The second group (Vac) was vaccinated with strain Mass H120 at three weeks of age and subjected to challenge at sixth weeks of age to the same field strain in EID<sub>50</sub> per bird dose 1 x 10<sup>8</sup> by intranasal and intraocular route. This group too was euthanized five days after challenge. Finally, a third group also comprising six birds was maintained as a negative control and was mock vaccinated and challenged with phosphate-buffered saline (PBS).

**Inhibition of ciliary activity:** For evaluation of tracheal ciliosstatic three fragments of approximately 1.5 mm of each portion, nine rings per bird, were analyzed. The rings were placed in a Petri dish containing Eagle culture medium with 10% fetal bovine serum. They were then analyzed on an inverted microscope, observing the degree of integrity and preservation of the ciliary movement of the tracheal epithelial cells, in accordance with Andrade et al. (1982); Cook et al. (1999); Yoshida et al., 1985. The tracheal ciliary activity were classified in scores from 0 to 3.

**Histopathology:** Fragments of kidney and portions from the upper, middle and the lower part of the trachea of approximately 0.5 cm were collected on the fifth day after challenge. They were fixed in 10% buffered formaldehyde solution for 1 day and processed by the standard histological procedure. They were embedded in paraffin wax and cut into 4 mm sections (Stevens, 2007). All of the sections were stained with haematoxylin and eosin and were examined by light microscopy for lesions due to IBV infection or untreated slides for use in immunohistochemistry.

The samples of trachea were assigned scores. A score of zero identifies the absence of injury, while cases with mild, moderate and severe injury were classified as one, two and three, respectively. Morphological characteristics were observed for loss of cilia and epithelial cells, degeneration of the epithelium and glands depletion, inflammatory infiltration and epithelial hyperplasia.

For kidney damage, injury was considered mild when there was mild tubular dilation with minimal infiltration of lymphocytic cells in the interstitial lumen. Moderate histological lesions were defined as the presence of small foci of necrosis and tubular dilatation with moderate inflammatory infiltrate. Severe lesions are defined as the presence of large foci of acute necrosis with tubular dilatation and severe inflammatory infiltrate around tubules as described by Nakamura et al. (1991) and Chen et al. (1996). Tissue sections were randomly photographed with a light microscope (Eclipse Nifocam, Nikon, Japan).

**RNA isolation and Real Time RT-qPCR:** The total RNA of tracheal and kidney tissue samples were extracted with TRIZOL Reagent® (Invitrogen, USA) and then cDNAs were obtained in the RT with a Superscript III Kit (Invitrogen, USA), as described previously. The cDNA samples were submitted to real time quantitative PCR for the absolute quantification of viral load, and this technique was conducted as recommended Okino et al. (2013), except that the primers described by Wang and
Tsai (1996) were used in place of HV+ and HV- primers. A linear regression was determined plotting the logarithmic values of the number of copy of plasmid DNA containing the insert of gene S1 against the cycle threshold (Ct) values, in order to convert the Ct values from tissue samples into S1 gene copy number (Okino et al., 2013).

Immunohistochemistry: IHC was performed on kidney and tracheas. The general procedure was initiated by deparaffinization of the sections followed by two baths in xylol. Then the samples were rehydrated in solutions of decreasing alcohol. Antigen retrieval was performed by heat, using a pressure chamber (Pascal, Dako, USA). Following this, the endogenous phosphatase was blocked through the addition of 200μL blocking solution commercial Dual Endogenous Enzyme Block (Dako, USA) for 20 min. The blocking of nonspecific reactions was done with Protein Block (Dako, USA). Incubation was performed with the primary antibody anti-IBV hyperimmune polyclonal antiserum, produced by our laboratory in goats, against the recombinant nucleoprotein of infectious bronchitis virus for 2 hr at 37°C in a humid chamber. Reaction was developed with an Envision-HRP Kit and EnVision™ G 2 System / AP, RB / Mo (Dako, USA). Counter-staining was made with Harris hematoxylin and the slides were mounted with Entellan (Merck). Between each of the steps, the slide was rinsed with a solution of distilled water and Tris HCl buffer solution, pH 7.4. The negative control technique was performed with Tris-HCl buffer, instead of the primary antibody, and another set of slides of the birds kept as the negative control of infection.

Statistical analysis: Results were expressed as Mean±SEM. Data were analyzed statistically using GraphPad Prism software version 5.0 for Windows (GraphPad Prism Software Inc., San Diego, CA, USA). The non-parametric test (Mann Whitney test) was used to determine the statistical significance and p<0.05 was considered statistically significant.

RESULTS

Clinical signs and gross pathology: All birds except those from the infection control group, showed sneezing and tracheal and bronchial rales with moderate hoarseness. Two to three days after challenge, four birds in the unvaccinated and challenged group were weak and depressed and one bird died. Only one bird in the vaccinated and challenged group had the same symptoms. At 4 dpi both groups showed increase in water consumption and slight watery diarrhea. At necropsy, morphological changes were observed in the kidneys of both the vaccinated and unvaccinated groups, as well as edematous and pale spots. Mucus and only mild congestion were present in the tracheas of both vaccinated and unvaccinated groups. Slight hyperaemia with catarhal exudate was seen in the trachea in unvaccinated group. There were no changes in other organs.

Inhibition of ciliary activity: The test of inhibition of ciliary activity was measured in three fragments of skull portions, medial and distal. Ciliostasis scores were very similar between the two challenged groups, with no significant difference between them (Fig. 1a).

Histopathology: In the tracheas, the histological changes were more evident in animals of the unvaccinated group; five out of the six birds showed moderate desquamation of ciliated cells (+), epithelial hyperplasia and degeneration of the mucus-producing cells and infiltration of heterophils and lymphocytes. The vaccinated group had only mild (+) infiltration of heterophils in the lamina propria, epithelial hyperplasia and moderate desquamation of ciliated cells across the group (Fig. 1b).

With respect to renal histopathology, all birds of the vaccinated and unvaccinated groups showed intense multifocal nephritis (+++) with lymphoplasmacytic infiltration, especially in the distal collecting tubules. We also observed mild necrosis of renal epithelial cells (Fig. 2a), deposition of urate crystals and proteinaceous material in the vaccinated group. In the unvaccinated group, moderate necrosis and epithelial degeneration was observed in four of the six birds (Table 1).

Real time RT-qPCR: The software generated a standard curve by plotting the cycle threshold (Ct) against the values of each dilution of the standard reference strain. The values were expressed as Log10 S1 gene copy number. All tracheas and kidneys analyzed were positive for the presence of the S1 gene of IBV, except the control group. Figure 3a shows greater presence of viral RNA in the trachea of the group non-vaccinated (p<0.01). This result was similar in renal analyzing samples (Fig. 3b) which contained greater number of viral copies in non-vaccinated group (p <0.05). Taking into account the numbers of copies of viral RNA present in the trachea and kidneys from non-vaccinated group, one notable difference was observed between them, in which the viral load in the kidneys was larger than the trachea (p <0.05).

Immunohistochemistry: The presence of IBV antigen was detected in all tissue samples from trachea and kidney. The cytoplasm of epithelial cells of the mucosa and lamina propria of the trachea were labeled and also the cilia left over. More intensive staining of viral antigen tracheal was noted within the group non-vaccinated, as
shown in Table 1. In kidney is shown in the presence of IBVPR-05 antigen was detected in renal tubular epithelium by IHC (Fig. 2b). Vaccinated group and non-vaccinated showed the same score of IBV-labeling by IHC in the tubular epithelial cells of the kidney (Table 1).

**DISCUSSION**

To our knowledge, this is the first report characterizing a Brazilian field isolate, previously classified in a S1 variant genotype, as a nephropathogenic pathotype. Since the first description of IBV in Brazil reported by Hipólito in 1957, antigenic and phylogenetic analyses have revealed, in the last two decades, IBV genotypes and serotypes in Brazil differing from those identified in North America, Europe, Asia, and Australia (DiFabio, 1993; Villarreal et al., 2007; Montassier et al., 2008; Montassier et al., 2012; Abreu et al., 2008). However, these isolates have not been tested to determine their pathotype, neither their protectotype with regard to the conventional vaccine strains used in Brazil.

The results of this study were surprising because of the marked nephropathogenicity exhibited by this Brazilian IBV variant isolate. After challenge with this isolate, birds in the vaccinated group developed symptoms common to IBV infection in the same intensity as the non-vaccinated group. The same relationship was seen in necropsy examinations of these birds. The primary difference was due to the death of one bird in the non-vaccinated group.

By comparing the scores of inhibition of tracheal ciliary activity of vaccinated and non-vaccinated groups, no difference in tracheal protection status was observed. Besides, the scores of histopathology in the trachea showed that the damage caused by the Brazilian variant strain IBVPR05 was higher in the non-vaccinated group than in vaccinated birds, which showed moderate inflammatory infiltration and epithelial degeneration.

In the kidneys, no difference was recorded in the intensity of nephritis, when vaccinated and non-vaccinated groups were compared. The kidney lesions caused by the IBVPR05 strain were similar to those described in previous reports, which characterized IBV nephropathogenic strains (Albassam et al., 1985; Ziegler et al., 1997-2000). Both experimental groups had, at 5 dpi, abundant renal lesions with diffuse lympho-histiocytic infiltration between the tubules, and adjacent to the distal tubules and the collecting ducts. Additionally necrosis and desquamation of tubular epithelial cells were detected in the kidney samples. Our finding with regard to the presence of acute interstitial nephritis at 5 dpi, further characterize this variant isolate as a nephropathogenic pathotype. Similar renal lesions were reported for an IBV field isolate from Egypt, which induces prominent lesions in the kidneys, at 5 and 7 dpi in birds vaccinated with H120, despite this Egyptian isolated has 97% homology on the nucleotide sequence of S1 gene from Massachusetts strain M41 (Abdel-Moneim et al., 2006).

Contrary to the Egyptian IBV isolate, genetically or antigenically different IBV strains can not have cross-immunity, and, consequently, the existing vaccine strains do not induce efficient protection for different organs of the chicken host against most of the heterologous strains or isolates (Ignjatovic and Sapats, 2000; Liu et al., 2006; Liu et al., 2009). Thus, other hypothesis for the low vaccine protection elicited by the H120 attenuated strain to the kidney tissues in this study, could be related to the fact that the IBVPR05 has relevant differences in its amino acid sequence of S1 glycoprotein with regard to H120 strain. Therefore, the degree of cross-protection tends to decrease as decreasing the extent of the identity of the amino acid sequences of S1 glycoprotein from two different strains of IBV (Cavanagh et al., 1997). IHC used in this study was able to detect the presence of viral antigen in both trachea and kidney samples from challenged birds. The IBV antigen was found in the cytoplasm of epithelial cells and also in the lamina propria of the trachea and in the cytoplasm of tubular epithelial cell. This finding further confirmed the results of histopathology with regard to the predominant IBV replication site in the kidneys. This site coincided with the distal tubules and collecting ducts described by Chen et al. 1996 by characterizing the pathogenicity of a nephropathogenic IBV field isolate. The IHC technique applied here also showed high sensitivity and specificity, as well as other protocols previously applied for antigen detection in different tissues (Naqi, 1990; Nakamura et al., 1991; Kapczynski et al., 2002; Benyeda et al., 2010). The results of RT-qPCR showed that the viral loads were higher in the kidney than in the trachea, for both vaccinated and non-vaccinated groups. Such finding reinforces the nephropathogenic character of this Brazilian IBV isolate. The results of RT-qPCR also showed that there was significant difference in the mean
number of S1 gene copy in the tracheal samples of vaccinated group and that showed by non-vaccinated group (P< 0.01). Likewise, the mean viral load detected in the kidneys from birds of non-vaccinated group was higher, and showed a significant difference compared to that observed in the vaccinated group (P<0.05).

Although there is a significant difference in the number of S1 viral gene copy in the kidney samples from vaccinated and non-vaccinated group, the vaccinated group still demonstrated a high viral load, and its kidney lesions were indistinct from those recorded in non-vaccinated birds, except for the epithelia degeneration. This indicates that the greater viral load in this organ could be related to the development of renal lesions. Like in infections by other coronaviruses, it is known that the IBV S1 glycoprotein is largely responsible for the tissue tropism of an IBV strain (Kuo et al., 2000). This glycoprotein plays a critical role in the viral infectivity and contains the antigenic determinants that induce the formation of neutralizing antibodies. Thus, the variation in the composition of amino acids located in some regions of the S1 glycoprotein could result in change in tissue tropism, virulence and antigenicity of a given IBV strain and constitutes the main strategy for IBV escapes from the host defense mechanisms (Cavanagh et al., 1997; Keeler et al., 1998; Cook et al., 1999).

Thus, the genetic alterations in the composition of S1 glycoprotein of IBVPR-05 resulted in a marked predilection for infection of epithelial cells which do not belong to the respiratory tract, possibly through an escape mechanism in the host, which in the case of this variant increased the tropism for kidney epithelial cells, and this led to the development of nephritis.
Fig. 2(a-b): (a) Photomicrograph of the kidney of a bird from vaccinated group showing the characteristic lymphoplasmacellular interstitial nephritis (black/white arrow), and mild degeneration of renal tubular epithelial cells (black arrow). Hematoxylin-eosin. (b) IBV antigen detection by immunohistochemistry in renal tubular epithelium of a bird from non-vaccinated group (black arrow). Note the specificity of immunostaining for IBV in the cytoplasm of the tubular epithelium associated to inflammatory cells. Scale bar = 50 μm.

This study assessed the immune-protection status induced by the conventional Massachusetts IBV strain (H120) against this Brazilian variant isolate. The results indicate that there was a better vaccine protection in the trachea due to lower pathogenicity of this Brazilian variant isolate for this organ compared to the kidney. A drastic drop in the number of S1 viral gene copy was detected in the tracheas of vaccinated birds, but not in the kidney samples of these birds. In fact, although there is a significant reduction in the number S1 gene viral copy and lesions in the kidneys of vaccinated birds, they still showed a severe nephritis.

The best protection against virulent challenge with nephropathogenic IBV strain is usually achieved by vaccination with homologous serotype strain (Lambrechts et al., 1993; Pensaert and Lambrechts, 1994). Due to the increasing isolation and identification of nephropathogenic IBV variants, including the Brazilian isolate IBRPR-05, it would be interesting to test this variant strain as a vaccine against this and others nephropathogenic Brazilian isolates, and compare to the status of cross-immunity induced by the reference Massachusetts vaccine strain.

Fig. 3(a-b): Detection of viral load (Log_{10} S1 gene copy number) by RT-qPCR in trachea (a) and kidney (b) samples collected from vaccinated (Vac), non-vaccinated (Non Vac) and control groups of chickens experimentally infected or not (Control group) with Brazilian variant IBVPR-05 isolate, at 5 dpi. A. Statistical differences between Vac and Non Vac groups are represented by ** (p<0.01) and * (p<0.05).
In conclusion, the IBVP05 variant isolate is characterized in this study as a nephropathogenic strain and based in its partial cross-immune protection with H120 Massachusetts strain, this virus must be classified in a different proteotype. This indicates also the importance of performing biologic analysis besides the genotyping for the Brazilian IBV variant isolates, in order to determine the efficacy of current IBV vaccines against these viruses arent to implement more effective control measures.

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Ethics Committee and Biosafety: This study was approved by the Institutional Ethics and Animal Welfare Committee (Comissão de Ética no Uso de Animais - CEUA, UNESP, process number 011497/11).

REFERENCES


