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

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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 protectotype 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 pathogen 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 (Dhinakar 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

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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 protectotypes (Dhinakar and Jones, 1996). 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; Felippe *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 investigate and characterize 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 protectotype 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_{50}) 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 $1 \times 10^{4.0}$ EID_{50} 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_{50} per bird dose $1 \times 10^{4.0}$ 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 mocked vaccinated and challenged with phosphate-buffered saline (PBS).

Inhibition of ciliary activity: For evaluation of tracheal ciliostasis 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); Yashida *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 0.5cm 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. Then 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 Moticam, 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 quantitive 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 trachea. 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 200uL 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 bronchiolar 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 catarrhal 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 Log_{10} 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

Table 1: Histopathology and IBV antigen detection by immunohistochemistry in tracheal and renal samples from vaccinated and non-vaccinated chickens challenged with the Brazilian variant IBVPR-05 isolate

Tissue	Trachea			Kidney		
Lesion	Epitelial deciliation	Heterophil infiltration	IBV antigen	Epitelial degeneration	Heterophil infiltration	IBV antigen
Non Vac[c]	++[a]	++	++[b]	++	+++	++
Vac	+	+	+	+	+++	++[a]

[a]Mean of the severity index: -, no change; +, mild; ++, moderate; +++, severe.
[b]Mean number of antigen positive cells per microscopic field IBV: -, no antigen; +, 1 to 4; ++, 5 to 10; +++, over 10.
[c]Experimental groups.

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.*, 2006). 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
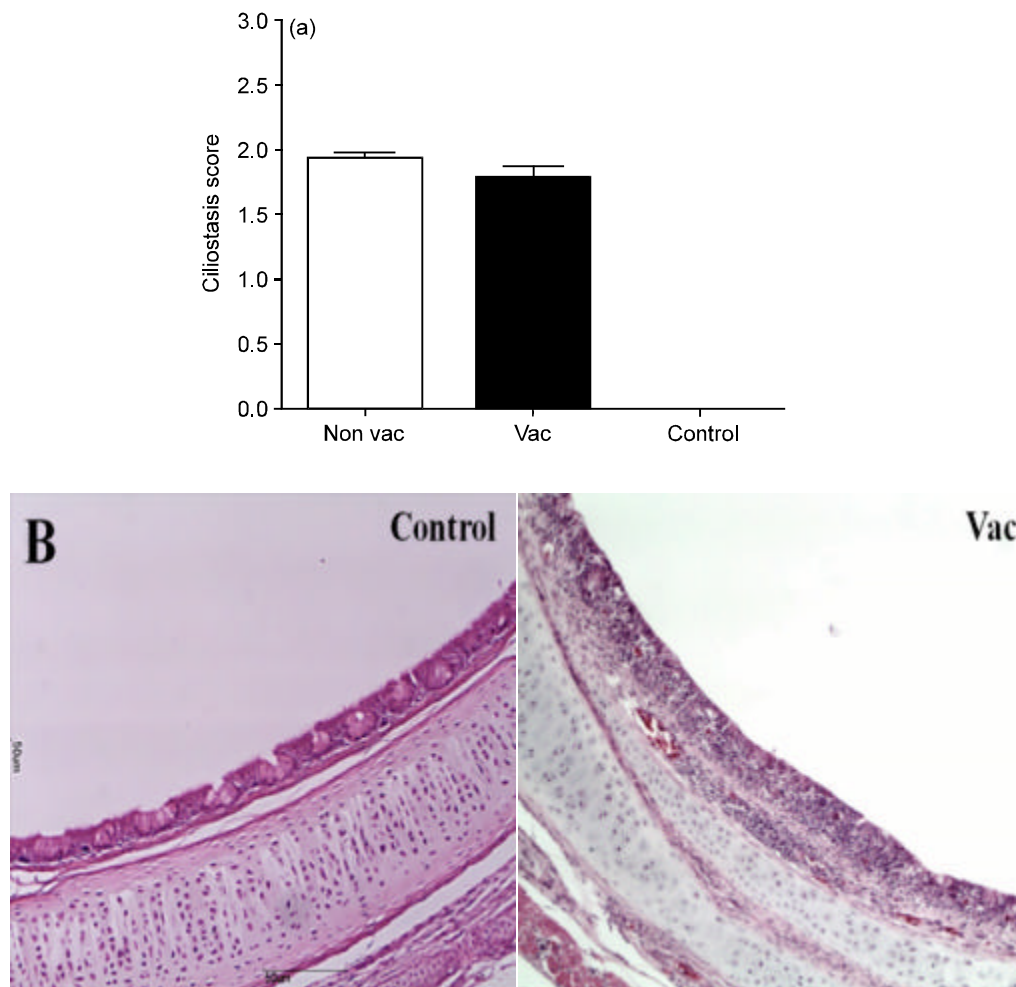
Fig. 1: Mean scores of the ciliary activity test and histopathological representation. (A) Score of ciliary stasis caused by IBVPR-05, there was no significant difference between the groups vaccinated and unvaccinated (p>0.05). (B) Focuses of infiltration of inflammatory cells and hyperplasia in the trachea from the vac birds (+) and no lesions for mock group.

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.
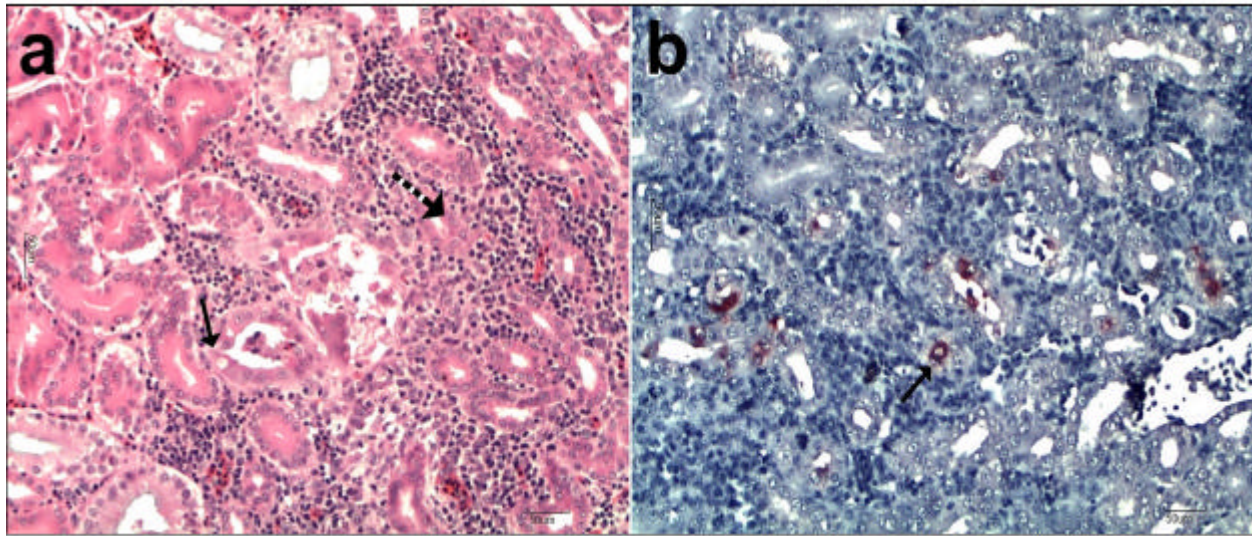
Fig. 2(a-b): (a) Photomicrograph of the kidney of a bird from vaccinated group showing the characteristic lymphoplasmacellular interstitial nephritis (black/white arrow), and mild degeneration of renal tubular epithelial cells (black arrow), Hematoxylin-eosin. (b) IBV antigen detection by immunohistochemistry 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.
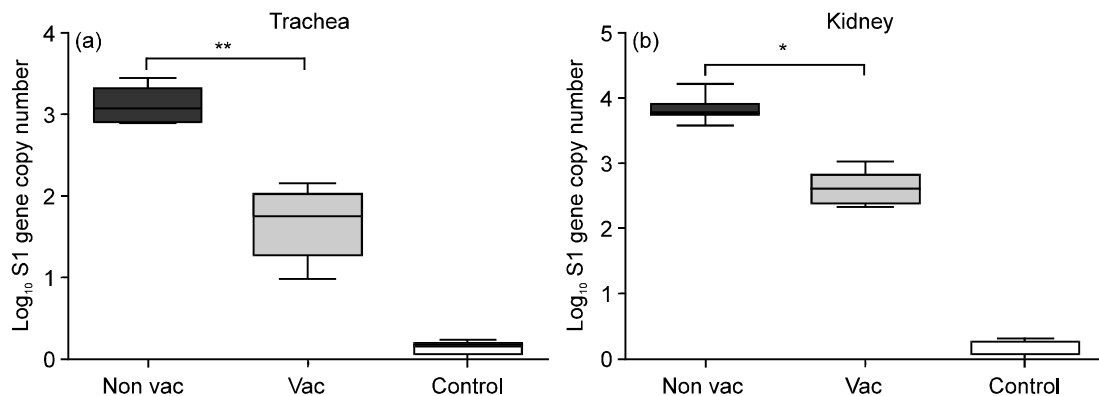

Fig. 3(a-b): Detection of viral load (Log_{10} S1 gene copy number) by RT-qPCR in trachea (a) and kidney (b) samples collected from vaccinated (Vac), non-vaccinated (Non Vac) and control groups of chickens experimentally infected or not (Control group) with Brazilian variant IBVPR-05 isolate, at 5 dpi. A. Statistical differences between Vac and Non Vac groups are represented by ** ($p<0.01$) and * ($p<0.05$).

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.

In conclusion, the IBVPR05 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 protectype. 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 ant to implement more effective control measures.

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 011467/11).

REFERENCES
Abdel-Moneim, A.S., M.F. El-Kady., B.S. Ladman and J.Jr. Gelb, 2006. S1 gene sequence analysis of a nephropathogenic strain of avian infectious bronchitis virus in Egypt. Virol. J., 3: 78.

Abreu, J.T., J.S. Resende., R.B. Flatschart., A.V. Folgueras-Flatschart., A.C. Mendes. and N.R. Martins, 2006. 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.

Albassam, N.R., R.W. Winterfield and H.L. Thacker, 1985. Comparison of the nephropathogenicity of four strains of infectious bronchitis virus. Avian Dis., 30: 468-476.

Andrade, L.F., A.P. Villegas., O.J. Fletcher. and R. Laudencia, 1982. Evaluation of ciliary movement in tracheal rings to assess immunity against infectious bronchitis virus. Avian Dis., 26: 805-814.

Benyeda, Z.S., L. Szeredi., T. Mato´., T. Su¨ Veges., G. Balka., Z. Abonyi-To´TH., M. Rusvai and V. Palya, 2010. Comparative Histopathology and Immunohistochemistry of QX-like, Massachusetts and 793/B Serotypes of Infectious Bronchitis Virus Infection in Chickens. J. Comp. Path., 143: 276-283.

Cavanagh, D., M.M. Ellis and J.K.A. Cook, 1997. Relationship between variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection. Avian Path., 26: 63-74.

Cavanagh, D., 2005. Coronaviruses in poultry and other birds. Avian Path., 34: 439-448.

Cavanagh, D. and S. Naqi, 2003. Infectious bronchitis. In: Calnek B.W., Barnes H.J. and Beard C.W. (ed.), Diseases of Poultry. 11th ed. Iowa State University Press, Ames, pp: 101-119.

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

Chen, B.Y., S. Hosi., T. Nunoya and C. Itakura, 1996. Histopathology and immunohistochemistry of renal lesions due to infectious bronchitis virus in chicks. Avian Path., 25: 269-283.

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

Cook, J., 2002. Coronaviridae. In: Jordan, F., Pattison, M., Alexander, D., Faragher, T. (Eds.), Poultry diseases. WB Saunders, New York, pp: 298-306.

Dhinakar Raj, G. and R.C. Jones, 1996. Protectotypic differentiation of avian infectious bronchitis viruses using an *in-vitro* challenge model. Vet. Microbiol., 53: 239-252.

Dhinaker Raj, G., and R.C. Jones, 1997. Infectious bronchitis virus: immunopathogenesis of infection in the chicken. Avian Path., 26: 677-706.

DiFábio, J., 1993. Bronquite infecciosa das galinhas: aspectos clínicos e controle da BI no Brasil. In: Conferência Apinco de ciência e tecnologia avícolas, Santos, SP. Anais, Campinas : FACTA, 1993, p:1-8.

Felippe, P.A.N., L.H.A. da Silva, M.M.A.B. Santos, F.R. Spilki and C.W. Arns, 2010. Genetic Diversity of Avian Infectious Bronchitis Virus Isolated from Domestic Chicken Flocks and Coronaviruses from Feral Pigeons in Brazil Between 2003 and 2009. Avian Dis., 54: 1191-1196.

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

Hipólito, O., 1957. Isolamento e identificação do vírus da bronquite infeciosa das galinhas no Brasil. Arq. Esc. Sup. Vet. (MG)., 10: 131-63.

Ignjatovic, J. and S. Sapats, 2000. Avian infectious bronchitis virus. Rev. Sci. Technol., 19: 493-508.

Kapczynski, D.R., H.S. Sellers., G.N. Rowland and M.W. Jackwood, 2002. Detection of in ovo-inoculated infectious bronchitis virus by immunohistochemistry and in situ hybridization with a riboprobe in epithelial cells of the lung and cloacal bursa. Avian Dis., 46: 679-685.

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

Kuo, L., G.J. Godeke, M.J. Raamsman, P.S. Masters, P.J. Rottier, 2000. Retargeting of coronavirus by substitution of the spike glycoprotein ectodomain: crossing the host cell species barrier. J. Virol., 74: 1393-1406.

Lambrechts, C., M. Pensaert and R. Ducatelle, 1993. Challenge experiments to evaluate cross-protection induced at the trachea and kidney level by vaccine strains and Belgian nephropathogeni c isolates of avian infectious bronchitis virus. Avian Path., 22: 577-590.

Liu, X.L., J.L, Su, J.X, Zhao and G.Z, Zhang, 2006. Complete genome sequence analysis of a predominant infectious bronchitis virus (IBV) strain in China. Virus Gen, Secaucus. 38: 56-65.

Liu, S., X. Zhang, Y. Wang, C. Li, Q. Liu, Z. Han, Q. Zhang, X. Kong and G. Tong, 2009. Evaluation of the protection conferred by commercial vaccines and attenuated heterologous isolates in China against the CK/CH/LDL/97I strain of infectious bronchitis coronavirus. Vet. J., 179: 130-136.

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

Montassier, M.F.S., L. Brentano, L.J. Richtzenhain, H. Montassier, 2012. Molecular analysis and evolution study of infectious bronchitis viruses isolated in Brazil over a twenty-one-year period. Proceedings of the 7th International Symposium on Avian Corona and Pneumoviruses and Complicating Pathogens; 2012; Wetter, Germany. Druckerei Schoreder., 1: 19-30.

Nakamura, K., J.K.A. Cook, K. Otsuki, M.B. Huggins, and J. Frazier, 1991. Comparative study of respiratory lesions in two chicken lines of different susceptibility infected with infectious bronchitis virus: histology, ultrastructure and immunohistochemistry. Avian Path., 20: 241-257.

Naqi, S.A., 1990. A monoclonal antibody-based immunoperoxidase procedure for rapid detection of infectious bronchitis virus in infected tissues. Avian Dis., 34: 893-898.

Okino, C.H., A.C. Alessi, M.F.S. Montassier, A.J.M. Rosa, X. Wang and H.J. Montassier, 2013. Humoral and Cell-Mediated Immune Responses to Different Doses of Attenuated Vaccine Against Avian Infectious Bronchitis Virus. Viral. Immunol., 26: 259-267.

Pensaert, M. and C. Lambrechts, 1994. Vaccination of chickens againsta Belgiann ephropathogenics train of infectious bronchitis virus B 1648 using attenuated homologous and heterologous strains. Avian Path., 23: 631-641.

Reed, L.J and H. Muench, 1938. A simple method of estimating fifty per cent end points. Am. J. Hyg., 27: 493-497.

Stevens, A., 2007. The haematoxylins. In J.D. Bancroft & A. Stevens (Eds.), Theory and Practice of Histological Techniques, 6th edn Edinburgh, Churchill Livingstone, pp: 107-118.

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.

Wang, C.H. and C.T. Tsai, 1996. Genetic grouping for the isolates of avian infectious bronchitis virus in Taiwan. Arch Virol., 141: 1677-1688.

Yashida, S., S. Aoyama, K. Sawaguchi, N. Takahashi, Y. Iritani and Y. Hayashi, 1985. Relationship between several criteria of challenche-immunity and humoral immunity in chickens vaccinated with avian infectious bronchitis vaccines. Avian Pathol., 14: 199-211.

Ziegler, A.F., B.S. Ladman, P.A. Dunn, A. Schneider, S. Davison, P.G. Miller, H. Lu, D. Weinstock, M. Salem, R.J. Eckroade, and J. Gelb Jr., 1997-2000. Nephropathogenic infectious bronchitis in Pennsylvania chickens 1997-2000. Avian Dis., 46: 847-858.