

## Experimental infection of inbred BALB/c and A/J mice with Massachusetts and Brazilian strains of infectious bronchitis virus (IBV)

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**Abstract** The ability of avian coronaviruses to replicate in mice was investigated to investigate interspecies transmission. Two inbred mouse strains (BALB/c and A/J) with different genetic backgrounds were inoculated with the avian coronavirus strains Mass and BR-I and monitored for at least 10 days. Analysis of viral RNA, histopathological examinations, immunohistochemistry and serology were performed. After virus inoculation, neither clinical signs nor evident gross lesions were observed. Viral RNA, histopathological changes, and viral nucleoprotein were observed in the lung, trachea and sinus of all inoculated mice. Our study demonstrates the importance of elucidating the epidemiology of coronaviruses, including in rodents that are pests in poultry production.

**Keywords** Virus · Mice · Avian · Pneumonia

Coronaviruses are a large family of viruses belonging to the order *Nidovirales*, family *Coronaviridae*. Recently, the discovery of novel coronaviruses resulted in the division of

the family *Coronaviridae* into two subfamilies: *Torovirinae* and *Coronavirinae*. The subfamily *Coronavirinae* is further split into four genera: *Alphacoronavirus*, *Beta-coronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* [9]. Members of the genera *Alphacoronavirus* and *Beta-coronavirus* are found mainly in mammals, in which they can cause mild or severe diseases [5]. This group includes the recently identified Middle East respiratory syndrome coronavirus [4]. Members of the genera *Gammacoronavirus* and *Deltacoronavirus* are found in birds and mammals [14, 18, 19]. The ancestors of the different genetic groups have not been identified.

Gammacoronaviruses have been detected in many Galliformes and non-Galliformes bird species over the past years [2]. Some experimental evidence suggests that coronaviruses are not limited to replication in a single host, and this is especially true of those that infect mammals [20]. Indeed, close contact of animals of different species in domestic environments and wildlife markets as well as close contact with humans may allow interspecies jumping and could subsequently pose risks of further genetic changes if the virus adapts to human hosts, as has occurred in the case of SARS [3] and H5N1 virus [17]. In the intensive poultry industry, poultry are increasingly exposed to pathogens of other birds and mammals and vice versa. An experimental study showed that bovine coronavirus (BCV) is pathogenic for 1-day-old turkey poults [10]. In this context, the present study aimed to evaluate whether avian coronaviruses can replicate, are pathogenic, and induce an immune response in mice.

Two inbred mouse lineages, BALB/c (MHC haplotype: H-2<sup>d</sup>) and A/J (MHC haplotype H-2<sup>a</sup>), were used for the experiments. The animals, 6- to 8-week-old specific-pathogen-free females, were purchased from CEMIB (Multidisciplinary Centre for Biological Investigation,

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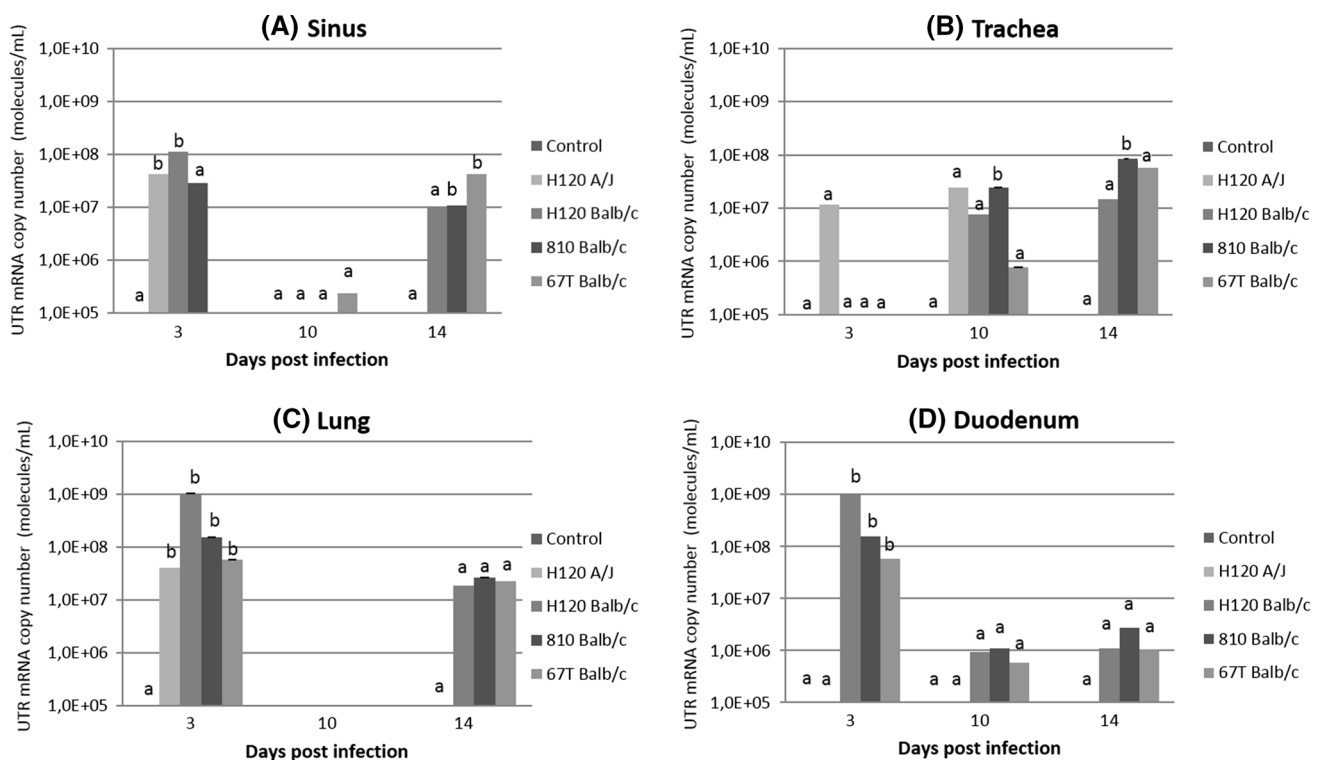
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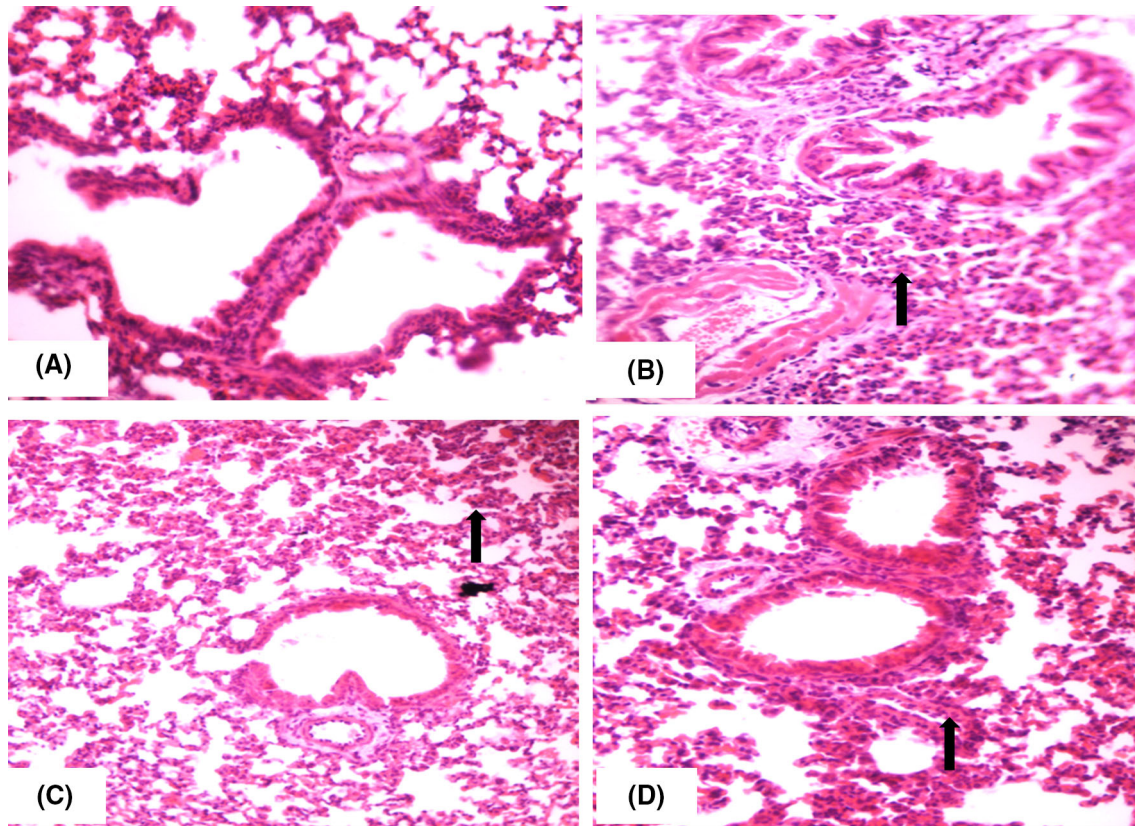
**Fig. 1** Detection and quantification of viral RNA in organs of BALB/c and A/J inbred mice after inoculation with different IBV strains at different time points. A, sinus; B, trachea; C, lung; D, duodenum. Mean  $\pm$  standard error at time points with no common superscript

differ significantly ( $P < 0.05$ ). Differences were only observed when more than one mouse was detected by the test. The limit of detection was 105.38 copies per mL

UNICAMP, Brazil). Mice were maintained under specific-pathogen-free conditions, and manipulations were performed according to the Brazilian Ethics Community guidelines and approved by the local ethics committee (CEUA-UNICAMP no. 2746-1, May, 21, 2012). Two IBV strains isolated previously [6], named 810 (BR-I) and 67T (Mass), and one vaccine strain (H120-Mass) (Bio-Bronk-Vet-H120, Laboratório Biovet, Vargem Grande Paulista, Brasil) were used. Thirty-six BALB/c mice were divided into four groups: a control group and three groups inoculated with virus (strains 67T, 810 or H120). Six A/J mice were inoculated with the H120 vaccine strain, and six A/J mice were kept as a control group. Mice were anesthetized and inoculated intranasally with 50  $\mu$ L of a suspension containing  $10^6$  EID<sub>50</sub> of IBV per mouse according to previous studies [16]. BALB/c and A/J mice were monitored daily for two weeks for weight loss and survival.

Subsequently, real-time RT-PCR (RRT-PCR) was employed to detect viral RNA in the sinus, trachea, lung, and duodenum. Tissues were homogenized in 400  $\mu$ L of ice-cold Dulbecco's modified Eagle medium (Life Technologies, Carlsbad, USA), and supernatants were harvested after centrifugation for 10 min at  $1000 \times g$  at 4  $^{\circ}$ C. Viral RNA from tissue suspensions were purified using a

QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RRT-PCR targeting the 5' untranslated region (5'UTR) of IBV was used [1]. The copy number was calculated using a standard curve, and beta actin was used as an internal control as described previously [7]. One-way analysis of variance and Student's t-test were carried out to compare differences among groups, and the differences were considered significant at  $P < 0.05$ . Histopathological evaluation was performed to assess whether the mice displayed any histopathological lesions related to infection. Trachea, lung, and duodenum of inoculated BALB/c mice were collected at 3 and 10 dpi and fixed in 4 % paraformaldehyde for 12 h at room temperature. The specimens were treated with xylene, dehydrated in graded ethanol, embedded in paraffin, and cut into 5- $\mu$ m-thick sections. Histopathological changes were evaluated in sections stained with hematoxylin and eosin (H&E). Additionally, the slides were subjected to immunohistochemistry for detection of virus in the tissues. Immunostaining was performed according to a previous study [11]. After treatment with Triton X-100 (#93443, Sigma Aldrich, St. Louis, USA) to permeabilize the cells, the tissue sections were incubated for 12 hours with a 1:20 dilution of a



**Fig. 2** Microscopic lesions in lungs of inoculated BALB/c mice. A) Control mice showing diffuse interstitial pneumonia. B) Severe interstitial pneumonia in mice inoculated with the H120 strain at 3 dpi. C) Moderate interstitial pneumonia induced by strain 810 at

3 dpi D). Mice inoculated with strain 67T had moderate interstitial pneumonia, diffuse oedema, and peribronchiolar mononuclear infiltration at 3 dpi. Arrowheads show interstitial pneumonia (haematoxylin and eosin staining; magnification, 40X)

monoclonal primary antibody targeting the IBV nucleoprotein (no. nAB90926, Abcam, Cambridge, UK). The slides were then incubated with a 1:20 dilution of goat anti-IgG biotin conjugate (no. B7264, Sigma Aldrich, St. Louis, USA). After washing, each slide was treated with a solution of avidin and biotinylated peroxidase. The reaction was visualized by adding a solution containing the substrate ( $H_2O_2$ ) and the chromogen diaminobenzidine (DAB). The sections were counterstained with hematoxylin, mounted, and examined using an optical microscope (Zeiss).

After blocking the tissue sections, a 1:20 dilution of a monoclonal primary antibody targeting the IBV nucleoprotein (no. nAB90926, Abcam, Cambridge, UK) was added, and the sample was incubated for 12 hours; After incubation, the slides were incubated with a 1:20 dilution of goat anti-IgG biotin conjugate (no. B7264, Sigma Aldrich, St. Louis, USA).

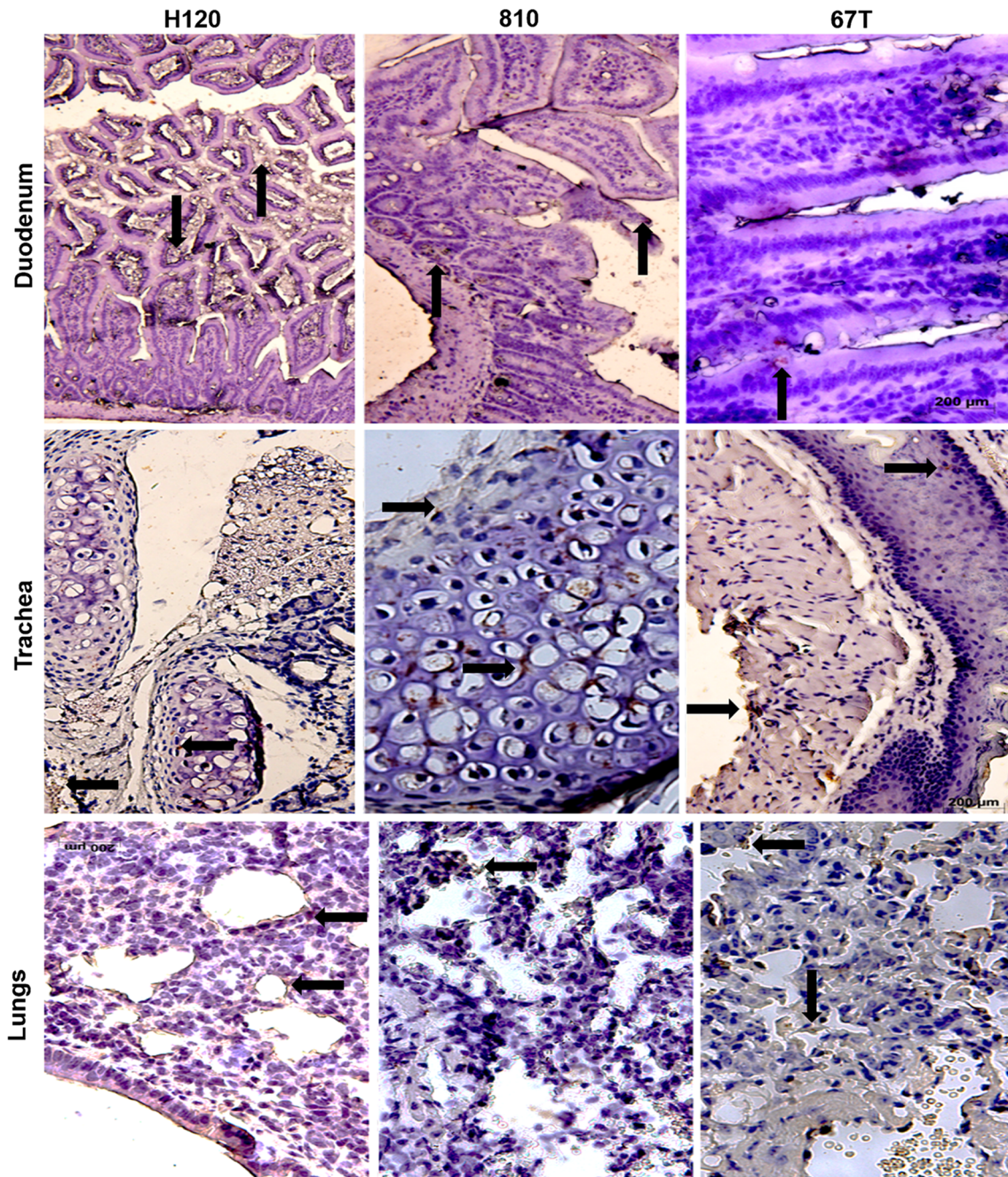
To determine whether IBV induced a humoral immune response in the mice, sera were collected at 3 and 10 dpi from all of the inoculated mice and tested using a

commercial competitive ELISA (IBVC-2P, Id Vet, Montpellier, France).

Although coronavirus infection usually results in weight loss in mice, no clinical signs or significant changes in weight were observed in the mice of either lineage during the entire experiment. In all cases, gross examination revealed normal tissue morphology with no noticeable gross lesions (data not shown).

After inoculation with virus, viral RNA was detected at different time points in the sinus, trachea, lung, and duodenum of mice of both inbred lineages (Fig. 1). In the sinus, the viral RNA level was significantly higher at 3 dpi in the A/J and BALB/c mice inoculated with the H120 strain than in mice inoculated with the 67T strain. Viral RNA was detected in the 67T- and 810-inoculated mice at 14 dpi (Fig. 1A). In the trachea, viral RNA was also detected in the mice inoculated with the 810 strain at 10 dpi and in the mice inoculated with the 810 strain at 14 dpi (Fig. 1B). For both inbred mouse lines, viral RNA was detected in the lungs of animals inoculated with each of the virus strains at 3 dpi (Fig. 1C). Viral RNA was also





**Fig. 3** Viral nucleoprotein in duodenum, trachea, and lungs of inoculated mice. Viral nucleoprotein was detected (arrows) in lamina propria and intestinal glands of the duodenum at 3 dpi in mice inoculated with the H120, 810, and 67T strains. Viral nucleoprotein was detected (arrows) at 3 dpi in the tracheal epithelium of mice

inoculated with the H120, 810, and 67T strains. Viral nucleoprotein was detected (arrows) in alveolar epithelial cells at 3 dpi in mice inoculated with the H120, 810, and 67T strains. No positive staining was seen in uninfected mice. Bars, 200  $\mu$ m

detected in the duodenum of all inoculated BALB/c mice at 3 dpi (Fig. 1D).

Mice inoculated with H120 virus had a large accumulation of mucus in the trachea at 3 dpi, but this was not observed until 10 dpi in the mice inoculated with strain 810. Mucus accumulation was not evident in the inoculated

mice from the 67T group. Interestingly, nucleoprotein was detected in all of the inoculated groups at 3 dpi (Fig. 3), but not at 10 dpi. Intense perialveolar and perivascular infiltration was observed in the lungs of all inoculated groups at 3 dpi when compared to the control group (Fig. 2A-D). Furthermore, the intense infiltrate was



substantially reduced at 10 dpi in the mice infected with 67T or H120, whereas the mice infected with the 810 virus showed persistent inflammatory infiltrate at 10 dpi. The viral nucleoprotein was detected in the lungs of all inoculated mice at 3 dpi (Fig. 3), especially in the mice inoculated with 67T virus, whereas the labeling decreased at 10 dpi. Moreover, in the duodenum, the increase in goblet cells with greater mucus production was more pronounced in mice inoculated with strain 810 than in the other groups at 3 dpi, but this was drastically reduced at 10 dpi. Nucleoprotein was also detected at 3 dpi in all of the inoculated mice; whereas this signal was no longer detectable at 10 dpi mice (Fig. 3). In addition to RNA viral detection by RRT-PCR, histopathological changes and changes in immunohistochemistry were observed in different tissues in the inoculated mice.

Specific antibodies to IBV were detected at 3 dpi in the BALB/c mice inoculated with H120, 810, or 67T, with percentage inhibition above 40 %:  $40.34 \pm 0.22$ ,  $74.97 \pm 0.04$  and  $42.62 \pm 0.17$ , respectively. The percentage inhibition value in the A/J mice inoculated with H120 was 30.54 % at 3 dpi, which corresponded to a suspicious outcome. No specific antibody was detected at 10 dpi in either mouse lineage.

In this study, we investigated whether avian gamma-coronavirus strains could replicate in a mammal model. Interestingly, viral RNA and histopathological changes in different organs were more evident in inoculated BALB/c mice, suggesting that these mice are more susceptible than A/J mice to avian coronavirus infection. BALB/c mice have been shown to be susceptible to experimental infection with avian paramyxoviruses and recombinant avian paramyxoviruses [11, 12]; but other studies have shown that A/J and C57BL/6 mice were more susceptible to infection with bovine respiratory syncytial virus than BALB/c mice [15] and that no difference was observed after infection with mouse hepatitis virus [13]. In our study, all tissue changes correlated to the detection of viral RNA by RRT-PCR or viral nucleoprotein by immunostaining, suggesting that virus replication had occurred, although virus isolation was not attempted. Viral nucleoprotein was strongly detected at 3 dpi, suggesting that the presence of virus correlated with severe histological changes. Those findings are in accordance with an acute inflammatory state [8]. Additionally, viral RNA, histopathological lesions and viral antigen were also more pronounced at 3 dpi than at 10 dpi, suggesting that replication occurred for a short time. Low levels of antibodies specific for IBV were detected at 3 dpi, suggesting slight IgM production, which is typically associated with the primary immune response. Nonetheless, no specific antibody was detected at 10 dpi, suggesting that this antibody response was not important given its short duration.

Our study demonstrates the importance of elucidating the epidemiology of coronaviruses that can infect a broad spectrum of hosts, including rodents that are pests in poultry production. Further studies should be performed to investigate whether rodents near poultry farms can carry avian coronavirus.

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