The Viral Distribution and Pathological Characteristics of BALB/c Mice Infected With Highly Pathogenic Influenza H7N9 Virus

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Abstract

Background: The highly pathogenic Influenza H7N9 virus is believed to cause multiple organ infections. However, there have been few systematic animal experiments demonstrating the virus distribution after H7N9 virus infection. The present study was carried out to investigate the viral distribution and pathological changes in the main organs of mice after experimental infection with highly pathogenic H7N9 virus.

Methods: The mice were infected with A/Guangdong/GZ8H002/2017(H7N9) virus via nasal inoculation. Some mice were killed at 2, 3, and 7 days after infection. The other mice were used to observe their illness status and weight changes. The characteristics of viral invasion were analysed using reverse transcription polymerase chain reaction and viral isolation. The pathological changes of the main organs were observed using haematoxylin and eosin staining and immunohistochemistry.

Results: The weight of mice infected with A/Guangdong/GZ8H002/2017(H7N9) virus increased slightly in the first two days. However, the weight of the mice decreased sharply in the following days by up to 20%. All the mice had died by the 8th day after infection and showed multiple organ injury. The emergence of viremia in mice was synchronous with lung infection. On the third day after infection, except in the brain, the virus could be isolated from all organs (lung, heart, kidney, liver, and spleen). On the seventh day after infection, the virus could be detected in all six organs. Brain infection was detected in all mice, and the viral titre in the heart, kidney, and spleen infection was high.

Conclusion: Acute diffuse lung injury was the initial pathogenesis in highly pathogenic H7N9 virus infection. In addition to lung infection and viremia, the highly pathogenic H7N9 virus could cause multiple organ infection and injury.

Background

Virus infection is a serious human health problem, and influenza virus is one of the major pathogenic viruses [1, 2]. Over the past 100 years, there have been many influenza epidemics, with the Spanish flu outbreak in the early 20th century being the worst. In the past 20 years, there have also been several serious influenza infection outbreaks. In 1997, high pathogenic H5N1 avian influenza virus crossed the species barrier to infect humans [3]. To date, the H5N1 outbreak has caused more than 800 infections, with more than 400 deaths, with a mortality rate of more than 50%. In 2009, the H1N1 pandemic appeared in the American continent. Since 2009, the H1N1 pandemic has spread worldwide, killing more than 200000 people. In 2013, the novel H7N9 avian virus started to infect humans, causing more than 1500 infections, with a fatality rate as high as 40% [4]. In 2017, the H7N9 virus mutated and changed to a highly pathogenic H7N9 virus. Since the first case of highly pathogenic H7N9 infection, these highly pathogenic H7N9 strains have caused human infection in 8 provinces in China, and 32 human cases were found to be caused by highly pathogenic H7N9 virus, with a mortality rate of around 50% [5]. At the

same time, the influenza virus is still changing, different viruses are still emerging, and a novel H7N9 virus might appear again, causing new threats to human health.

Studies have shown that H7N9 constantly adapts to its human host and increases its affinity for human receptors [4]. Many clinical and epidemiological studies have shown that H7N9 displays obvious family aggregation, indicating that close contact between people might lead to H7N9 infection [6–10]. Once H7N9 increases its adaptability to human receptors to acquire human transmission ability, it will lead to an influenza epidemic and mass infection [11]. The H7N9 virus already poses a significant potential threat to humans. Mortality could increase because of mutations in highly pathogenic H7N9. Therefore, on the one hand, we should study the affinity of the virus, and on the other hand, we should also study the pathogenic characteristics of the virus, especially its multiple organ distribution and pathological characteristics after virus infection.

Many influenza viruses have been reported have acquired an ability for extrapulmonary infection[12, 13]. The most frequently described clinical entities are viral myocarditis and viral encephalitis[14, 15, 1]. The H1N1 virus has been confirmed to cause viremia, which led to brain infection. H5N1 has also been confirmed to cause viremia [16]. Viremia was detected in fatal cases, but was absent in nonfatal cases [17]. Intense inflammatory responses and a high viral load are central to influenza H5N1 pathogenesis. H5N1 viruses were proven to have extra-pulmonary pathology, which contributes to morbidity [18–21]. H7N9 virus RNA was also detected from the plasma of many patients, but with no correlation with clinical outcome. H7N9 virus RNA could be detected in urine and faeces [22]. We have confirmed the presence of viremia in highly pathogenic H7N9 infections. The initial infection site of highly pathogenic H7N9 is the lung. Lung infection is the main manifestation, and it will eventually develop to multiple organ failure [23]. Patients infected with H7N9 virus developed cytokine storms and viremia, and died from multiple organ failure. Whether multiple organ failure is an inflammatory response caused by lung infection, or a later viral, multi-organ infection is unclear, and solving this problem would guide treatment. There is currently limited clinical and pathological research on H7N9 virus infection, and there have been few systematic animal experiments to prove the virus distribution after H7N9 virus infection [24]. To answer the above questions, we designed experiments in mice infected with highly pathogenic H7N9 viruses to clarify the viral distribution and pathological changes to important organs after viral infection.

Methods

Animal experiment ethical statement

Twelve female specific-pathogen-free (SPF) BALB/c mice, aged 6–8 weeks old, were purchased from the Experimental Animal Center of Zhejiang Province, China. All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Zhejiang Province. The study was approved by the Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine, (Ethical approval No. 2017.402-1). All experiments with H7N9 virus were performed in a bio-

safety level 3 laboratory of the First Affiliated Hospital, Zhejiang University School of Medicine (Registration No. CNAS BL0022).

Cells and viruses

The Madin-Darby canine kidney cell line (MDCK) was obtained from the ATCC (Rockville, MD, USA). The cell line was propagated in growth medium comprising Dulbecco's modified Eagle's medium (DMEM; Cat#11965092, Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS; Cat#10100147, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C and 5% CO₂. The A/Guangdong/GZ8H002/2017(H7N9) virus used in this study was isolated from a patient in Guangzhou, China, in 2017 (GenBank: MF455313-455320). Virus stocks were propagated in the allantoic cavities of 9-day-old specific pathogen free (SPF) embryonated chicken eggs at 37 °C for 72 h. The allantoic fluid was harvested and tested using a haemagglutinin (HA) assay, in which 50 µl of allantoic fluid solution was double diluted at 1:2 with phosphate-buffered saline (PBS; Cat#20012500BT, Gibco, Grand Island, NY, USA) in a 96-well blood coagulation plate. Then, an equal volume of 1% chicken red blood cells were added, and observed at room temperature for 30–45 min. The highest dilution at which blood coagulation appears is the HA titre. Aliquots of the allantoic fluid containing the virus were stored at –80 °C until further use.

Determination of the virus TCID₅₀

MDCK cells were inoculated into 96-well cell culture plates at 3×10^4 cells/well (in 100 µl). The virus was diluted in viral growth liquid 10 times continuously, from 10^{-1} to 10^{-10} , and the last two rows were reserved for control. After cells grew into a single layer in the 96-well plate, the medium was discarded, the wells were washed once with sterile PBS, and the diluted virus (100 µl/well) was added to the wells, with each concentration infecting cells in four wells. A normal cell control well (virus free) was set. The 96-well plate was incubated at plate at 37 °C in a 5% CO₂ incubator for 2 h, and then washed two times with PBS. The normal control hole and the virus infection wells then received virus growth fluid (DMEM with 1% cow serum albumin; 100 µl/well). The plate was incubated at 37 °C in a 5% CO₂ incubator for 72 h. The HA assay was then used to identify positive or negative wells. The median tissue culture infectious dose (TCID₅₀) was calculated as we did in the previous study [25].

Virus inoculation

Mice were inoculated intranasally with 50 µl 10^6 TCID₅₀ A/Guangdong/GZ8H002/2017(H7N9) virus. The same volume of PBS was given to the mice in the control group. Mice were observed for signs of illness, weight loss, and death post-infection. Then, at 2, 3, and 7 days post-infection (dpi), some of the mice were sacrificed. Their serum and organs (lung, brain, heart, kidney, liver, and spleen.) were collected. Part of the organ was fixed in 10% buffered formalin, while the other part was used to isolate the virus and detect virus levels using quantitative PCR.

The histopathology of organ tissue

Organ tissue was prepared for haematoxylin eosin (HE) staining. Immunohistochemical (IHC) assays were also conducted. Paraffin sections of organs were de-waxed and then subjected to heat treatment in citrate buffer. Endogenous peroxidase activity was quenched using 0.3% H₂O₂ in methanol. Sections were blocked for 2 h with 3% bovine serum albumin (BSA; Cat#H1130, Solarbio, Tongzhou, Beijing, China) in PBS and incubated sequentially overnight at 4 °C with 1:200 dilution of polyclonal rabbit anti-H7N9 antibodies (Cat#GTX125989, GeneTex, Irvine, CA, USA) for 12 h. Antibody binding was detected using EnVision System reagents (Cat#K5007, DAKO, Glostrup, Denmark). All slides were counterstained with haematoxylin and eosin.

Isolation of the virus from mouse serum and organs

The serum samples collected at 2, 3, and 7 dpi were used to isolate the virus. About 100 µl of serum was injected into the allantoic cavities of 9-day-old SPF embryonated chicken eggs. The embryonated chicken eggs were cultured in an incubator at 37 °C for 72 h. The allantoic fluid was harvested and tested using an HA assay.

The tissue leachate was obtained as follows: One ml of sterile PBS was added to the frozen tissues in storage tubes, and then the tissue was cut using sterile scissors in the biosafety cabinet. The tubes were centrifuged at 500 g for 10 minutes. Then, 200 µl of the supernatant was used to isolate the virus. The virus separation of tissue leachate refers to the serum virus separation procedure.

Determination of the virus titre of organs

One ml of sterile PBS was added to the frozen tissues in storage tubes, and then the tissue samples were cut using sterile scissors in the biosafety cabinet. The tubes were centrifuged at 500 g for 10 minutes. Then, 200 µl of the supernatant was added with 800 µl Trizol to extract RNA. The amount of virus was then evaluated using quantitative real-time PCR. A H7N9 nucleic acid quantitative detection kit (Cat#Z-RR-0309-02) purchased from Zhijiang biological technology Co., Ltd. (Shanghai, China) was then applied. The steps were as follows: 19 µl of H7N9 nucleic acid PCR detection reaction mixture and 1 µl quantitative PCR enzyme were mixed by vortexing and then centrifuged at 500 g for several seconds. This mixture was added to the PCR reaction tube, together with 5 µl of the RNA sample, for a total reaction volume of 25 µl. The tube was covered, centrifuged briefly, and subjected to the following PCR conditions: 40 cycles of 45 °C for 10 minutes, 95 °C for 15 seconds, and 60 °C for 60 seconds. The relative quantity of H7N9 virus was determined by the cycle threshold (Ct) value.

Statistical analysis

Statistical analyses of the weight data and the survival rate were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

The weight change and survival status of the mice

We observed illness, weight loss, and death in the mice after H7N9 challenge. During the first two days, the mice infected with A/Guangdong/GZ8H002/2017(H7N9) showed mild illness, with a minor decrease in appetite and activity. The weight of the mice increased slightly over the first two days. However, the weight of the mice decreased sharply during subsequent days, reaching nearly 20% weight loss (Fig. 1A). The health status of the mice gradually deteriorated over the following days. The mice presented a relatively acute clinical process, and showed inactivity, ruffled fur, and poor appetite post-infection. By day 7 post-infection, more than 64% of the mice had died (Fig. 1B). The remaining mice presented with severe signs of respiratory disease, including respiratory distress and further lack of appetite, and nearly 20% weight loss. All the mice were dead by day 8 post-infection (Fig. 1B). The mice in normal control group were healthy during the entire observation period with no obvious weight loss or illness.

Histopathology of organs

Histopathology of lung tissue was evaluated by HE staining. The extent and characteristics of the lesions varied at different dpi. The mice at 2 dpi showed a lesion with inflammatory cell infiltration, exudation, and intra-alveolar haemorrhage. The mice at 3 dpi had severe multifocal interstitial inflammatory hyperaemia and exudative pathological changes in the lung. The mice at 7 dpi had larger lesions in their lung tissue compared with those at 2 and 3 dpi, and showed fusion of multiple patchy lesions. At the same time, the intra-alveolar haemorrhage area was enlarged (Fig. 2A). Immunohistochemical staining of the lungs revealed various degrees of injury according to the dpi. Viral antigens could be detected in the lung, especially in the bronchiolar epithelium. The mice at 3 and 7 dpi suffered severe injury, with many viral antigens detected (Fig. 2A).

HE staining was also performed in brain tissue. The mice infected with A/Guangdong/GZ8H002/2017(H7N9) presented increasingly severe pathological changes with extended infection time. In the IHC staining of brains, large amounts of H7N9 viral antigens were detected in the brain at 7 dpi (Fig. 2B). The results suggested that the virus had infected the brain tissue.

The histopathology of heart tissue was also evaluated by HE staining. The extent and characteristics of the lesions varied according to the dpi. In the IHC staining of hearts, large amounts of H7N9 viral antigens were detected in the hearts at 3 and 7 dpi (Fig. 2C). The results suggested that the virus had infected the heart tissue from 3 day after infection.

HE staining was also performed in kidney tissue. The mice infected with A/Guangdong/GZ8H002/2017(H7N9) presented increasingly severe pathological changes with extended infection time. In the IHC staining of the kidney, large amounts of H7N9 viral antigens were detected at 3 and 7 dpi (Fig. 3A). The results suggested that the virus had infected the heart tissue from 3 day after infection.

Histopathology of liver tissue was also evaluated by HE staining. The extent and characteristics of the lesions varied by dpi. In the IHC staining of liver, H7N9 viral antigens were detected in the liver at 3 and 7

dpi (Fig. 3B). The results suggested that the virus had infected the liver tissue from 3 dpi.

HE staining was also performed in spleen tissue. The mice infected with A/Guangdong/GZ8H002/2017(H7N9) presented increasingly severe pathological changes with extended infection time. In IHC staining of the spleen, the H7N9 viral antigens were detected in the liver at 3 and 7 dpi (Fig. 3C). The results suggested that the virus had infected the spleen tissue from 3 day after infection.

Virus isolation from mouse serum and organs

We isolated the H7N9 virus from the mouse serum. In the A/Guangdong/GZ8H002/2017(H7N9)-infected mice, live virus was detected persistently in the serum until death (Table 1). At 2 dpi, H7N9 virus was isolated from one of three mice. Virus could be isolated from mice at 3 and 7 dpi. H7N9 virus could not be isolated from the brain at 2 and 3 dpi, whereas the virus could be isolated from all mouse brains at 7 dpi. The H7N9 virus was not isolated from the heart at 2 dpi. At 3 dpi, virus could be isolated from the heart of one of three mice. At 7 dpi, virus was isolated from three of six mouse hearts. The H7N9 virus was not isolated from the kidney at 2 dpi. At 3 and 7 dpi, virus was isolated from the kidney of one of three mice and two of six mice, respectively. The H7N9 virus was not isolated from the liver at 2 after infection. At 3 dpi and 7 dpi, virus was isolated from the liver of one of three and one of six mice respectively. The H7N9 virus was not isolated from the spleen at 2 dpi. At 3 dpi and 7 dpi, virus was isolated from the spleen of one of three mice and two of six mice, respectively.

Table 1: The H7N9 virus isolated from serum and different tissues of mice

Tissue	Number of animals from which the virus was isolated		
	2 dpi (n = 3)	3 dpi (n = 3)	7 dpi (n = 6)
Lung	1	3	6
Brain	0	0	6
Heart	0	1	3
Kidney	0	1	2
Liver	0	1	1
Spleen	0	1	2
Serum	1	3	6
Dpi, days post infection.			

The amount of virus of mouse organs

Quantitative PCR was used to evaluate the amount of virus in mouse organs. The RNA level was represented by the Ct value. A Ct value > 38 was considered negative. The lower the Ct value, the higher the virus content. The RNA level was relatively low in the lungs of the mice at 2 dpi, but increased sharply at 3 dpi and again at 7 dpi (Table 2). The RNA level was relatively low in the brains of the mice at 2 and 3 dpi, but increased to a high level at 7 dpi. The RNA level was relatively low in the hearts of mice at 2 dpi. However, the virus content in the heart was relatively high in one of three mice at 3 dpi and in three of six mice at 7 dpi. The RNA level was relatively low in the kidneys of the mice at 2 dpi. However, the virus content in the kidney was relatively high in one of the mice at 3 dpi and in two of six mice at 7 dpi. The RNA level was relatively low in the livers of the mice at 2 dpi. However, the virus content in the liver was relatively high in one of three mice at 3 dpi and in one 1 of six mice at 7 dpi. The RNA level was relatively low in the spleens of mice at 2 dpi. However, the virus content in the spleen was relatively high in one of three mice at 3 dpi and in two of six mice at 7 dpi.

Table 2: Reverse transcription-polymerase chain reaction results in different tissues of each group of mice

Mouse group	Reverse transcription-polymerase chain reaction results in different tissues											
	Lung (H7)	Lung (N9)	Brain (H7)	Brain (N9)	Heart (H7)	Heart (N9)	Kidney (H7)	Kidney (N9)	Liver (H7)	Liver (N9)	Spleen (H7)	Spleen (N9)
2 dpi-1	34.30	34.48	38.84	36.17	36.24	33.80	33.03	32.36	32.77	31.17	31.80	31.68
2 dpi-2	30.58	31.41	33.65	31.11	36.90	32.52	37.71	34.15	37.23	34.25	34.08	31.25
2 dpi-3	35.42	36.27	34.40	32.87	34.23	35.91	33.74	32.87	39.84	34.07	34.91	33.05
3 dpi-1	28.36	29.11	33.59	32.71	36.30	35.32	34.78	33.67	33.08	31.28	34.35	33.66
3 dpi-2	30.41	30.30	30.58	30.26	36.88	32.36	35.03	33.38	31.50	29.32	38.71	33.74
3 dpi-3	18.43	19.96	33.60	33.23	29.16	30.06	30.73	30.80	24.01	25.71	26.49	28.25
7 dpi-1	16.25	18.85	17.20	18.99	34.60	35.14	34.13	32.42	34.67	31.61	32.09	29.15
7 dpi-2	14.66	17.40	21.39	23.20	31.04	32.35	31.04	26.72	33.24	31.79	33.98	32.57
7 dpi-3	16.63	19.11	19.87	21.07	32.92	33.65	32.42	31.68	30.81	30.47	34.38	34.19
7 dpi-4	15.47	18.48	16.67	18.26	28.25	30.92	34.73	33.68	33.24	32.99	34.23	32.26
7 dpi-5	20.08	22.98	21.29	22.66	29.20	30.05	33.55	34.04	34.61	33.38	33.11	31.64
7 dpi-6	17.42	19.64	20.82	22.45	26.08	28.07	31.10	28.40	30.78	29.28	33.64	33.96

Note: dpi: days post infection. The H7 and N9 gene levels are shown by the cycle threshold (Ct) value. A Ct value > 38 was considered negative.

Discussion

We have confirmed that viremia was associated with the high mortality caused by highly pathogenic H7N9. Lung infection is the main manifestation, and the virus eventually causes multiple organ failure. Multiple organ failure may have many causes, including a systemic inflammatory response resulting from pulmonary infection [26], but might also be caused by late multiple organ infection of the virus. In the present study, we found that the weight of mice infected with A/Guangdong/GZ8H002/2017(H7N9) virus sharply decreased from 3 dpi. Our result suggested that in the early time of infection (up to 2 dpi), lung lesions were few and the virus titre was low. During this early period, the other organs were not

infected. The first two days is a very important time window for early diagnosis and treatment. In a previous study, the early use of a neuraminidase inhibitor within 2 days of illness shortened the duration of viral shedding and improved survival in patients with H7N9 viral infection [27]. In addition, as the virus has changed, the time of antiviral use has been extended to fully control the infection [27]. Thus, in highly pathogenic H7N9 virus infection, antiviral treatment should be started as early as possible. We observed that on the third day after infection, the disease burden increased sharply, promoting the risk of multiple organ infection, which would require a longer period of antiviral therapy and might result in multiple organ injury.

In our previous study, the H7N9 virus was not lethal to mice [25]; however, all the mice infected with highly pathogenic H7N9 were dead by the 8th day after infection and had multiple organ injury. Heart, kidney, and liver injuries are common in influenza infection [28, 29, 13]. Moreover, the presence of influenza A (H1N1) in pericardial and myocardial tissues of patients with influenza was reported [30, 31]. These studies suggest that direct viral invasion is a potential cause of infection-associated cardiac injury. Heart changes have also been reported during H7N9 infection. In a previous study, among 321 patients, 203 showed evidence of cardiac injury [32]. Cardiac injury was associated with severe infections, acute respiratory distress syndrome (ARDS), and related mechanical ventilation settings [32]. The study proved that cardiac injury was associated with higher mortality during hospitalization [32]. In our study, heart infection was confirmed in mice. Heart infection might also be the cause of high rates of pathogenic H7N9 mortality. Therefore, we should closely monitor myocardial function in patients infected highly pathogenic H7N9.

In our study, during the late stage of infection, all the mice showed brain infection. Influenza-associated encephalopathy might be another important cause of death in patients with influenza [33]. Seasonal and pandemic H1N1 infections in 2009 were found to cause encephalopathy in patients [34, 35]. In previous studies, the highly pathogenic H7N9 virus exhibited enhanced virulence and extended viral tropism in mice compared with low pathogenic H7N9 viruses [36]. Meanwhile, the highly pathogenic H7N9 virus has been proven to replicate in brain tissues in animal models [36]. Given that the virulence of the H5N1 virus in mice correlates with its virulence in humans, it is reasonable to speculate that the mouse-lethal highly pathogenic H7N9 strain might also be more lethal in humans. If highly pathogenic H7N9 virus can cause brain infections in mice, it might also cause brain infections in humans. If a patient develops multiple organ injury and brain infection, they will require urgent intensive care treatment. In the intensive care unit, we should maintain organ function, strengthen antiviral treatment, and provide life support treatment. Such comprehensive treatment can save patients' lives [4, 37]. At the same time, we should pay attention to a patient's intracranial pressure and note any changes to their mental capacity [38].

Conclusions

In summary, influenza virus mutations increase infection rates in humans and may also lead to pathogenic enhancement. We should closely monitor the H7N9 virus because it might gain greater multi-organ infection capacity through mutations, leading to higher fatality rates. Acute diffuse lung injury is

the initial stage of pathogenesis in highly pathogenic H7N9 virus infection. In addition to lung infection and viremia, the highly pathogenic H7N9 virus can cause multiple organ infection and injury, which should receive more research attention. Therefore, early diagnosis, early use of drugs or neutralization antibodies to control the virus, and the prevention and control of multiple organ infection are very important.

Declarations

Ethics approval and consent to participate

All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Zhejiang Province. The study was approved by the Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine, (Ethical approval No. 2017.402-1).

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article

Competing interests

The authors declare that they have no competing interests.

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Author contributions

HY, NW, and LL designed the experiments. XW, ST, SY, YZ, LX, LC, and FL performed the experiments. XW and ST drafted the manuscript. All authors participated in revising the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

The weight change and survival curve of the experimental mice A: The weight change of the experimental mice. B: The survival curve of the experimental mice.

Figure 2

Histopathology of lung, brain, and heart tissue A: HE staining and immunohistochemical staining of the lungs of mice at 2, 3, and 7 days post infection. B: HE staining and immunohistochemical staining of the brains of mice at 2, 3, and 7 days post infection. C: HE staining and immunohistochemical staining of the hearts of mice at 2, 3, and 7 days post infection. The viral antigen is shown using a red arrow. HE: Haematoxylin and eosin staining; IHC: Immunohistochemical staining; dpi: days post infection.

Figure 3

Histopathology of kidney, liver, and spleen tissue A: HE staining and immunohistochemical staining of the kidney of mice at 2, 3, and 7 day post infection. B: HE staining and immunohistochemical staining of the livers of mice at 2, 3, and 7 days post infection. C: HE staining and immunohistochemical staining of the spleens of mice at 2, 3, and 7 day post infection. The viral antigen is shown using a red arrow. HE: Haematoxylin and eosin staining; IHC: Immunohistochemical staining; dpi: days post infection.