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Commercial vaccine against pseudorabies virus: A hidden health risk for dogs

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ABSTRACT

Pseudorabies virus (PRV) is considered as an infectious agent with a wide of host range, causing considerable economic losses in animal husbandry. Although the commercial vaccine against PRV plays an critical role in control of this disease in swine industry, the potential risk of commercial vaccines against PRV for other host is unclear. Here, we report that the commercial vaccine against PRV is a hidden health risk for dogs. We found that different attenuated PRV strains in commercial vaccines possess different tissue tropism, and that the attenuated PRV strains are lethal to dogs, and that the attenuated PRV strain possesses the ability to spread horizontally among the dogs. Collectively, our findings provide clues that the commercial vaccine against PRV is a hidden risk for dogs, even for the owner of pet dogs to take seriously.

1. Introduction

Pseudorabies (PR) disease is an economically critical viral disease of pigs worldwide (Mettenleiter, 2000). The causative agent of this disease is pseudorabies virus (PRV), which is also called suid herpesvirus 1 (SuHV-1). PRV is a member of the genus *Varicellovirus* of the subfamily *Alphaherpesvirinae* within the family *Herpesviridae* (Nauwynck et al., 2007).

Pigs have been confirmed as the primary host and reservoir of PRV (Marcaccini et al., 2008; Mettenleiter, 1996). Besides the pig, PRV has a wide spectrum of hosts, including cattle, sheep, dog, cat, even some avian species (Mettenleiter, 2000; Pomeranz et al., 2005). There are numerous reports about PRV infection in dogs either due to consuming raw meat or by direct contact with PRV-infected swine, indicating that dogs are highly susceptible for infection with PRV. In addition to its importance in animal husbandry, PRV has potential public health significance. In *vitro*, PRV can readily infect human cells in cell culture, indicating the zoonotic threat theoretically (Tischer and Osterrieder, 2010). Although it has been demonstrated that human beings are resistant against natural PRV infection (Jentzsch and Apostoloff, 1970), several suspected cases describing putative infection of humans with PRV were reported. The first suspected case of PRV infection in human was reported in 1914, but the detection of antibodies against PRV and the virus cultivation had failed. Later, another suspected case of PRV infection in human was reported in 1987, 3 patients exhibited central nervous system symptoms and produced positive antibodies against PRV (Mravak et al., 1987). A latest report describing the etiology of a human endophthalmitis case in southern China concluded that there is evidence supporting the role for PRV in human infection and disease of eyes, and PRV can infect human after direct contact with pig contaminants (Ai et al., 2018). Therefore, PRV can be considered as a potential zoonotic agent with a wide of host range.

Currently, more and more companion animals walked into human life. As the presence of companion animals (especially pet dogs) becomes increasingly ubiquitous in human life, the influence of the health of such animals on human health is growing. Considering the susceptibility of PRV to dogs and human and the intimate contact of dogs with human, the potential threat of PRV to public health security existed. However, the epidemiology and pathogenicity of PRV in dogs is still unclear. In the present study, we investigated the epidemiology in dogs in pig farms or pet hospitals, evaluated the pathogenicity and transmission of distinct PRV strains in dogs. Our findings provide a basis for

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the research into the mechanism behind the pathogenicity of PRV.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Animal Care Committee of South China Agricultural University (approval ID: SYXK-2014-0136). All study procedures and animal care activities were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China.

2.2. Vaccines, virus and animals

Two commercial live attenuated PRV vaccines, vaccine A (Bartha-K61 strain, Batch no.150137018) and vaccine B (HB98 strain, Batch no.170041056), were purchased from YEBIO Co., Ltd and Wuhan Keqian Animal Biological Co., Ltd, respectively. Both commercial vaccines against PRV are widely used in Chinese swine industry. DCD-1 strain (a highly pathogenic strain) was isolated and obtained in our laboratory. Eighteen 60-day-old beagle dogs, which were determined to be free of PRV by ELISA and PCR assay, were purchased from Guangdong National Beagles Resources Research Center (Approval ID: SCXK-2013-0007).

2.3. Antibody detection

A total of 19 serum specimens were collected from dogs in pig farms or pet hospitals in Guangdong province during the period from May 2017 to June 2018. Among these 19 serum specimens, 16 specimens were collected from companion dogs (including 1 samples collected from pre-mortal dogs that exhibited pruritus, dyspnea and muscle spasms, and 4 samples collected from dogs that died without showing any of typical symptoms), and other 3 specimens were collected from homeless dogs in pig farms. The anti-gB antibodies in the serum samples were detected using the PRV/ADV gB Antibodies Test Kit (Batch no. 99-09732, IDEXX) according to the manufacturer's instructions, and the anti-gE antibodies in the serum samples were detected using the Anti-gE Antibody ELISA Kit (Batch no. 180304, Keqian Corp.) according to the manufacturer's instructions.

2.4. Viral titers

The viral titers of PRV in the commercial vaccines were titrated using 50% tissue culture infective doses ($TCID_{50}$) as described previously (Verpoest et al., 2016). Briefly, 100 μL of the vaccines were diluted in 900 μL Dulbecco's modified Eagle's medium (DMEM, Invitrogen Corp.) with penicillin (100 IU/mL) and streptomycin (100 μg/mL), and filtered through 0.22-μm filters. The viral solution was serially diluted 10-fold in DMEM. A 100-μL aliquot of each diluted sample was added to the wells of multiple 96-well plates, followed by addition of PK15 cells at a density of 2×10^5 cells/mL. Cells were cultured for 3 days at 37°C in 5% CO_2. The culture wells with CPE were considered to be positive. Viral titers were determined according to the Reed and Muench method (Reed, 1938).

2.5. Challenge study

To assess the pathogenicity of commercial attenuated PRV vaccine in dogs, a total of twelve 60-day-old beagle dogs were randomly divided into four group (three dogs per group). The dogs in group I and group II were inoculated with PRV commercial vaccine Bartha-K61 and HB98, respectively, via the intramuscular inoculation route at a dose of $10^{5.0}$ $TCID_{50}$, which is same as the dose used for piglets. The dogs in group III were inoculated with PRV strain DCD-1 at a dose of $10^{5.0}$ $TCID_{50}$ as a positive infection control, and dogs in group IV were inoculated with PBS as a mock infection control. Dogs in different groups were raised in independent rooms. Clinical signs of disease and mortality were monitored daily. The blood samples and excretion were collected at 0, 3, 6, 9, 12 and 14 days post infection (dpi). Dogs in each group were euthanized till one of them presented with the nervous signs of disease or sudden death. The rest dogs were euthanized for tissue collection at 14 dpi. Tissues, including the brain, heart, liver, spleen, lung, kidney, pancreas, thymus, intestinal tract, esophagus, trachea and stomach, were collected for further pathological analysis. To assess whether PRV can be transmitted between dogs, a total of six beagle dogs were raised in the same room. Three dogs were inoculated with Bartha-K61 via the intramuscular inoculation route at a dose of $10^{5.0}$ $TCID_{50,}$ while other dogs were inoculated with PBS. Clinical signs of disease and mortality were monitored daily. The blood samples and excretion were collected at 0, 7, 14 and 28 dpi. All the dogs were euthanized till one of them presented with the nervous signs of disease or sudden death. Tissues were collected for further pathological analysis.

2.6. Virus detection in the excretion

Viral DNA of the excretion of oral cavity, nasal cavity and anus were extracted using TRIzol reagent according to the manufacturer's instruction (Invitrogen Corp.). The specific primer pairs 5′-GGACGGGC GCCACCCCAGACGGCTT-3′ (sense) and 5′-CAGACGTAGAAGCGGTCC CGCTCGG-3′ (anti-sense) targeting on the PRV gB gene were designed according to the previous publication (Tong et al., 2016). The PCR was performed to detect the presence of PRV.

2.7. Cytokine detection

The blood samples were collected from the fore leg vein and processed to collect sera for detection of cytokines. The pro-inflammatory cytokines IFN-β and TNF-α in the serum were determined using the Canine TNF-α ELISA Kit (Batch no. SU-B76147, MIBIO) and Canine IFN-β ELISA Kit (Batch no. SU-B76017, MIBIO) according to the manufacturer's instruction.

2.8. Quantification of viral loads

Viral DNA were extracted from the affected tissues using TRIzol reagent, and subjected to the quantitative real-time PCR (qRT-PCR) to determine the viral distribution in artificially challenged dogs. A specific primer set 5′-TGAAGCGGTTCGTGATGG-3′ (sense) and 5′-CCCCG CACAAGTTCAAGG-3′ (anti-sense) were designed based on the highly conserved sequence within the gB region of the PRV genome. The qRT-PCR was performed in a 20 μL volume containing 10 μL of 2 × SYBR Premix ExTaq Green mix (TaKaRa), 1 μL of DNA template, and a 0.5 mM concentration of specific primers. Thermal cycling parameters were as follows: 95°C for 5 min; 40 cycles of 95°C for 10 s, 56°C for 30 s, and 72°C for 30 s and 1 cycle of 95°C for 30 s, 60°C for 30 s, and 95°C for 30 s. All the samples were reacted in triplicate on the same plate. The analysis of qRT-PCR was carried out with a CFX96 Touch (Bio-Rad).

2.9. Histopathology

The fresh affected tissues were fixed in 10% neutral-buffered formalin, routinely processed, embedded in paraffin, sectioned (4-μm thick), and stained with hematoxylin and eosin (H&E) according to standard protocols. Pathological changes were examined by light microscopy.

2.10. Statistical analysis

Statistical analyses were performed with the GraphPad Prism (version 5.0) and expressed as means and standard deviation. The

significance of the differences between PRV-infected dogs and controls in temperature or body weight, and between PRV-infected dogs and controls in cytokine production was determined by Mann-Whitney test or analysis of variance, respectively. Differences between groups were considered significant when the *P* value was less than 0.05.

3. Results

3.1. PRV infection and shedding in dogs

It has been reported that dogs can be infected with PRV by consuming contaminated raw pork or offal (Quiroga et al., 1998), we speculated that PRV might be widespread in dogs. To test this hypothesis, we collected the serum specimens from dogs in the pig farms and pet hospitals, and then detected the anti-gB antibodies and anti-gE antibodies in the serum specimens, respectively. As a result, a total of 6 serum specimens were positive for PRV gB, but only 3 serum were positive for PRV gE, indicating that 3 dog were infected with gE-deletion PRV strain. Among the serum collected from pet hospitals, 5 serum specimens were positive for PRV gB, and 3 serum were positive for PRV gE. While among the serum collected from pig farms, only 1 serum were positive for PRV gB, and no serum were positive for PRV gE, indicating that only one dog in pig farms was ever infected with gE-deletion PRV strain (Table 1). All these data indicated that PRV infection exist in dogs in China.

3.2. PRV infection cause clinical manifestation in dogs

To assess whether the commercial attenuated PRV vaccine cause lesions in dogs, we inoculated 60-day-old beagle dogs with the commercial attenuated PRV vaccines via the intramuscular inoculation route. As a result, the death rate of dogs in the HB98-inoculated group and DCD1-inoculated group reached to 100%, while no death occurred in the Bartha-K61-inoculated group and mock-inoculated group during the experiment (Fig. 1a). Generally, in the DCD1-inoculated group, one dog died naturally at 2 dpi (46 h post infection), and other two dogs were euthanized at 3 dpi, following the presentation of anorexia, dyspnea, pruritus and vocalization. In the HB98-inoculated group, all the dogs were euthanized at 6 dpi, following the presentation of anorexia, dyspnea and pruritus. The dogs in Bartha-K61-inoculated group and the mock-inoculated group without any signs of disease were euthanized at the end of the experiment for further analysis.

Clinical observations indicated that the dogs inoculated with HB98 strain showed typical signs of PR, including pruritus, dyspnea, ataxia and muscle spasms. Similar clinical signs were observed in dogs inoculated with DCD1 strain. However, no typical signs of the disease were observed in the dogs of Bartha-K61-inoculated group compared with that of the mock-inoculated group. Interestingly, the virus were detected in the nasopharyngeal swab, anal swab and the buccal swab of the artificially challenged dogs at 3 dpi (Table 2), indicating the successfully inoculation of PRV in the experimental dogs.

The temperatures of the dogs in Bartha-K61-inoculated group had an analogous trend to that in mock-inoculated group, but showed a little fever on at 12, 13 and 14 dpi. High fever was observed at 2 dpi in DCD1-inoculated group, and at 4 dpi in HB98-inoculated group (Fig. 1b). The average body weight of the dogs in Bartha-K61-inoculated group had an analogous trend to that in mock-inoculated group. While there was no significant change in body weight in HB98-inoculated group, but a significant decrease in DCD1-inoculated group (Fig. 1c). All these data indicated that DCD1 strain and HB98 strain possess higher virulence than Bartha-K61 strain.

Table 1
Epidemiological survey of PRV in dogs.

Antibody	Pet hospitals		Pig farms	
	Positive	Negative	Positive	Negative
anti-gB	5	11	1	2
anti-gE	3	13	0	3

3.3. The attenuated PRV strains in commercial vaccine cause histopathological lesions in dogs

PRV has been confirmed to cause severe lesions in natural- or experimental-infected dogs (Zhang et al., 2015b). It was intriguing to analyze the pathogenicity of the commercial PRV vaccines in the dogs. Therefore, we performed the autopsy and recorded the gross abnormalities at necropsy. As a result, compared to the dogs in the mock-inoculated group, the Bartha-K61-infected dogs did not exert any clinical symptoms of disease at necropsy, while the HB98-inoculated dogs and DCD1-inoculated dogs presented with apparent lesions (Fig. 2a–h). Generally, the HB98-inoculated dogs showed the multifocal pulmonal hemorrhages in lung, focal hemorrhage in spleen, and congestion in the mesentery, but no lesions in brain (Fig. 2e). The DCD1-inoculated dogs presented with severe hemorrhages and congestion in brain, focal hemorrhage in the heart, liver, kidney, pancreas and spleen, and the punctate hemorrhage in stomach and intestinal wall (Fig. 2g & h), which are consistent with the previous reports. Interestingly, in this study, we observed focal ecchymoses in the lung of Bartha-K61-inoculated dogs at 14 dpi, this findings are contrary to the previous report that Bartha-K61 is not only safe for dogs, but also effective in inducing an immune response to the antigen it carries, indicating the feasibility and practicality of PRV strain Bartha-K61 as an approved vaccine (Yuan et al., 2008).

To further evaluate the pathogenicity of PRV in dogs, histopathological analysis was performed. Similar to the observations at necropsy, among the dogs in these group, the HB98- and DCD1-inoculated dogs presented with most severe lesions of multi-organs, while the Bartha-K61-inoculated dogs only presented with lesions in the brain and lung. Compared to the dogs in the mock-inoculated group, severe lymphohistiocytic perivascular infiltration of neuroparenchyma and gliosis were observed in the cerebrum of Bartha-K61-, HB98- and DCD1-inoculated dogs (Fig. 3a-d), the degeneration and necrosis of neurons occurred in the brainstem of HB98- and DCD1-inoculated dogs (Fig. 3e–h). The apparent pulmonary hemorrhage and congestion occurred in the lung of Bartha-K61-, HB98- and DCD1-inoculated dogs (Fig. 3i–l). While apparent hemorrhage only occurred in the liver, kidney, and pancreas of DCD1-inoculated dogs (Fig. 4).

3.4. Different PRV strains have different tissue tropism in dogs

To study on the distribution of distinct PRV strains in dogs, we detected the presence of Bartha-K61, HB98 and DCD1 strain in the organs of the dogs. As a result, virus were detected in all the organs of the DCD1-inoculated dogs, the dogs had much more viral loads of PRV genomes in the heart, liver, spleen, lung, kidney, intestinal tract, esophagus and pancreas compared with that of other both groups, but had much less viral loads of PRV genomes in the brain, stomach and trachea compared with that of HB98-inoculated group. On the contrary, the dogs of Bartha-K61-inoculated group had lower viral loads of PRV genomes than others, and no virus were detected in the liver, intestinal tract, trachea and pancreas in the dogs of dogs. Furthermore, no virus was detected in the liver of HB98-inoculated dogs (Fig. 5). All these data indicated that all these PRV strains have different replicate rate and tissue tropism.

3.5. PRV infection induces the pro-inflammatory cytokines production

Because the pro-inflammatory cytokines play important role in the host immune response against pathogen (Biron, 1998; Trevejo et al.,

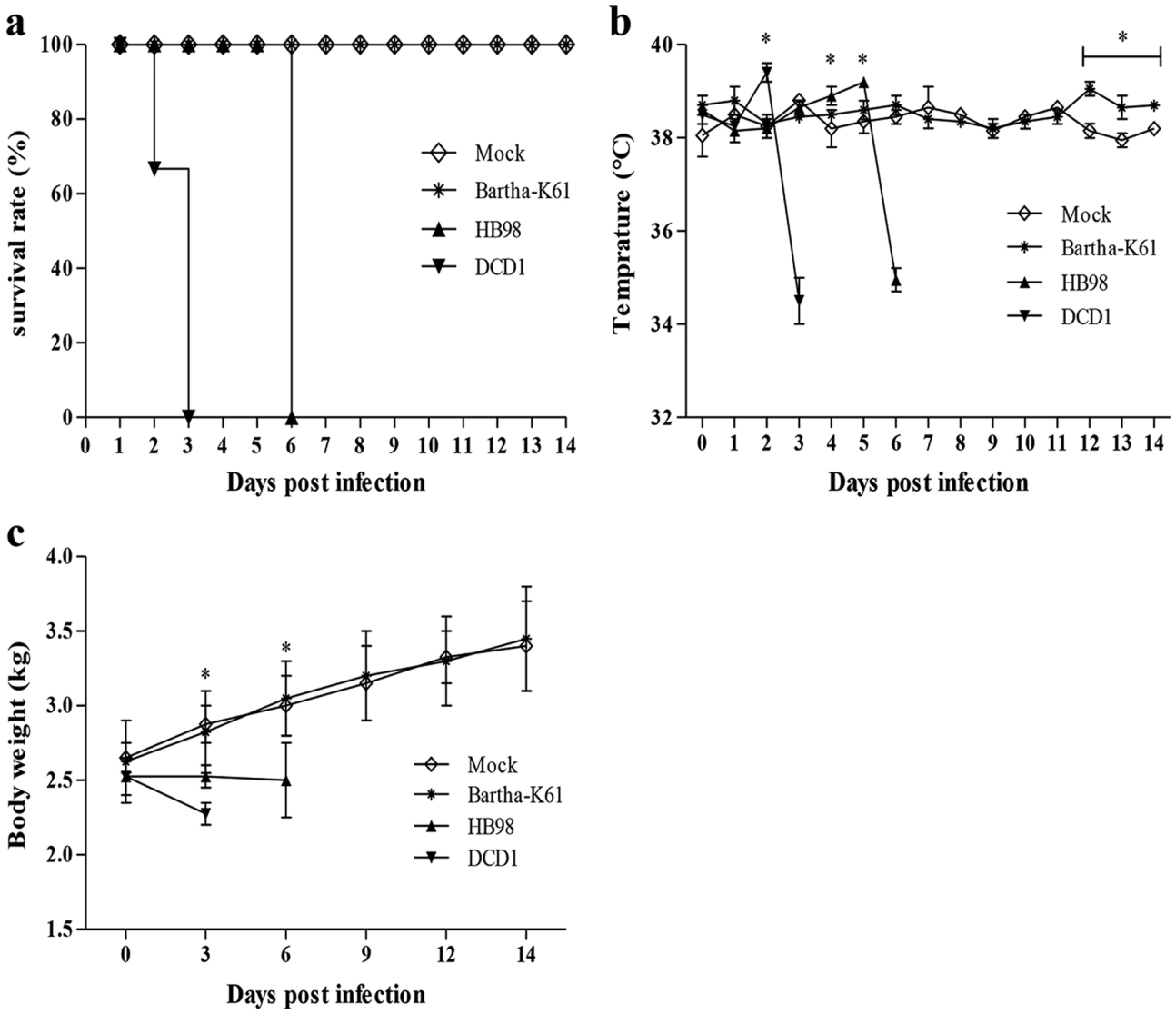

Fig. 1. Clinical signs of PRV-infected dogs. 60-day-old beagle dogs were infected with Bartha-K61, HB98, DCD-1 or PBS as a control. Clinical signs of disease and mortality were monitored daily. (a) Survival curves for each group. (b) Variation of mean rectal temperature in each group. (c) Body weight changes for each group.

Table 2
Virus detection in the excretion of artificially challenged dogs.

Group	Sample	Days post infection					
		0	3	6	9	12	14
Mock	Buccal swab	0/3	0/3	0/3	0/3	0/3	0/3
	Nasopharyngeal swab	0/3	0/3	0/3	0/3	0/3	0/3
	Anal wab	0/3	0/3	0/3	0/3	0/3	0/3
Bartha-K61	Buccal swab	0/3	3/3	3/3	3/3	3/3	3/3
	Nasopharyngeal swab	0/3	3/3	3/3	3/3	3/3	3/3
	Anal wab	0/3	3/3	3/3	3/3	3/3	3/3
HB98	Buccal swab	0/3	3/3	3/3	/	/	/
	Nasopharyngeal swab	0/3	3/3	3/3	/	/	/
	Anal wab	0/3	3/3	3/3	/	/	/
DCD1	Buccal swab	0/3	3/3	/	/	/	/
	Nasopharyngeal swab	0/3	3/3	/	/	/	/
	Anal wab	0/3	2/3	/	/	/	/

2001), we proposed that pro-inflammatory cytokines might be involved in PRV infection in dogs. Therefore, we measured the production of TNF-α and IFN-β by ELISA in the serum. As a result, HB98- and DCD1-inoculated dogs had much more TNF-α in the serum than controls during the infection period ($P < 0.05$), whereas Bartha-K61-inoculated dogs produced more TNF-α at 14 dpi ($P < 0.05$) (Fig. 6a). IFN-β are dominant during the early phase of viral infection. As expected, all the Bartha-K61-, HB98- and DCD1-infected dogs had more IFN-β than controls at the early phase, but no significantly difference after 3 days (Fig. 6b).

3.6. PRV can be transmitted among the dogs

Our previous study indicated that dogs can be infected with commercial vaccine against PRV. It was intriguing to evaluate the characteristics of PRV transmission in dogs. Therefore, we raised three PRV-free dogs and three Bartha-K61-inoculated dogs together, monitored the clinical signs of disease and mortality, and ended the experiment till the dogs presented with sudden death or nervous signs of disease. As a result, the experiment ended at 28 dpi, a total of four dogs (including three Bartha-K61-inoculated dogs and one contact-exposed dog) died without showing any of the typical symptoms during the experiment period, and other dogs were euthanized at 28 dpi. The death rate of Bartha-K61-inoculated dogs reached to 100% (Table 3), indicating that PRV Bartha-K61 strain is lethal to dogs. Clinical signs of this disease in Bartha-K61-inoculated dogs and contact-exposed dogs were monitored daily. However, no typical clinical symptoms of disease were observed

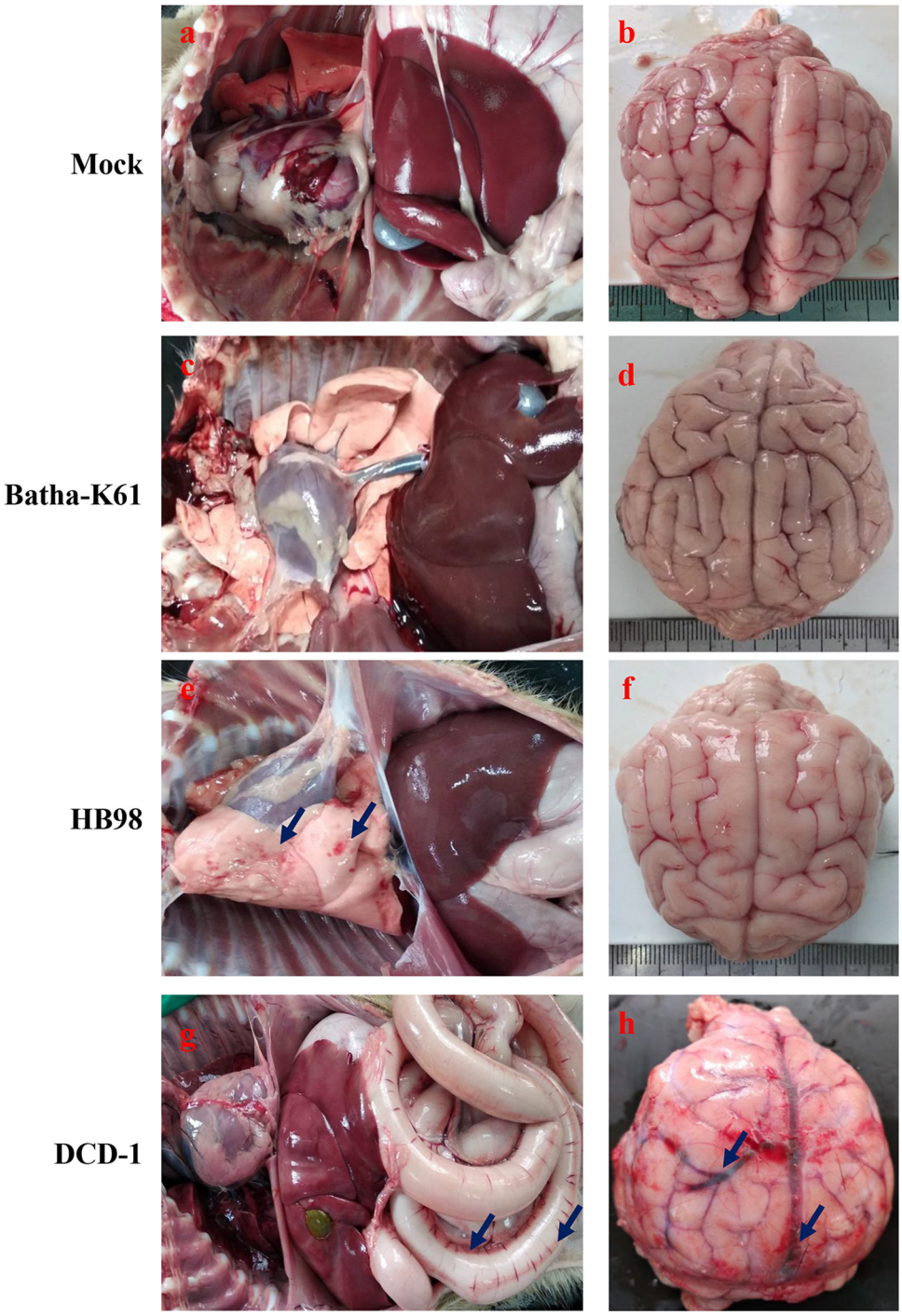

Fig. 2. Gross pathologic changes in PRV-inoculated dogs. 60-day-old beagle dogs were infected with Bartha-K61, HB98, DCD-1 or PBS as a control. All the dogs were euthanized at 14 dpi. The autopsy was performed, and the gross abnormalities were recorded at necropsy. (a& b) The necropsy symptoms of the mock-infected dogs. (c&d) The necropsy symptoms of the Bartha-K61-infected dogs. All the tissues seemed to be normal macroscopically. (e&f) The necropsy symptoms of the HB98-infected dogs. Multifocal pulmonal hemorrhages were observed (labeled with arrows). (g&h) The necropsy symptoms of the DCD1-infected dogs. The hemorrhages and congestion were observed in the brain (labeled with arrows).

during the experimental period, except for a little change of the temperature of the dead dogs before death (Fig. 7a). The body weight of Bartha-K61-inoculated dogs had an analogous trend to that in the contact-exposed group at the early infection stage, but the initially immunized as well as one of the contact-exposed dogs grew more slowly than the rest contact-exposed dogs at the late infection stage (Fig. 7b). At necropsy, focal ecchymoses were observed in the lung and spleen, multifocal hemorrhages were observed in the stomach and kidney (Fig. 7c–f). All these data indicated that Bartha-K61 strain possesses the pathogenicity in dogs.

Our previous study showed that Bartha-K61 can be replicate in the heart, spleen, lung, kidney, brain, esophagus and stomach, so we detected the viral loads for each Bartha-K61-inoculated and contact-exposed dogs. As expected, Bartha-K61 was detected in these organs with a high load (Fig. 8). Additionally, the Bartha-K61 were detected in the excretion of the contact-exposed dogs after 7 days, and isolated from all the contact-exposed dogs. All these data indicated that PRV can be transmitted between dogs.

4. Discussion

In China, the first case of PRV was documented in the 1947 in cats, and subsequently reported in cattle and swine (Sun et al., 2016). As the increase in the intensity of swine production in China, PRV had been spread widely in China in the 1980s. By early 1980s, PRV infection had been identified in 18 regions (14 provinces, 1 municipality and 3

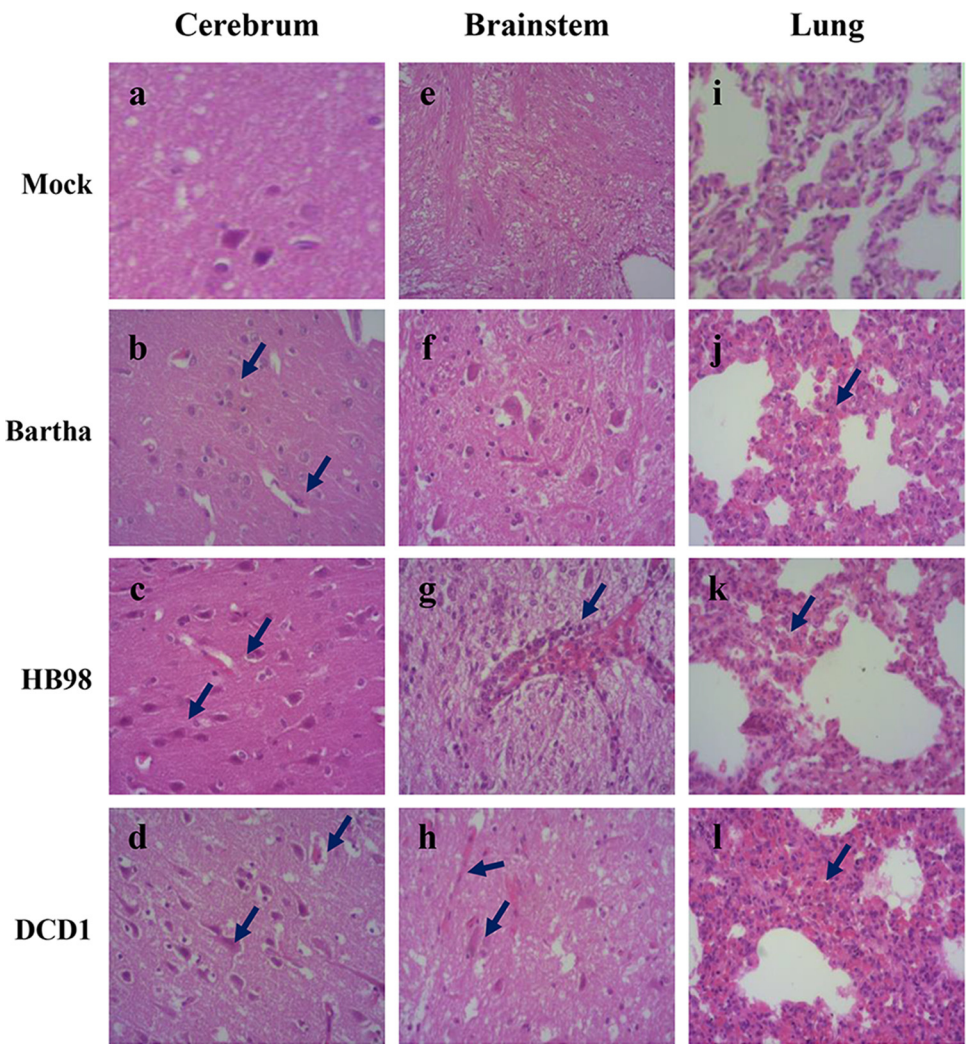

Fig. 3. Histological changes in dogs infected with PRV. Affected tissues of mock-, Bartha-K61-, HB98- and DCD1-infected dogs were fixed by immersion in 10% neutral-bufered formalin, routinely processed, embedded in parafn, sectioned (4 μm thick), and stained with hematoxylin and eosin. Histological changes were examined by light microscopy. (a–d) Histological changes of the cerebrum. The lymphohistiocytic perivascular infiltration of neuroparenchyma and gliosis were observed in the cerebrum of Bartha-K61-, HB98- and DCD1-inoculated dogs. (e–h) Histological changes of the brainstem. The degeneration and necrosis of neurons occurred in the brainstem of HB98- and DCD1-inoculated dogs, while no changes were observed in the brainstem of Bartha-K61-inoculated dogs. (i–l) Histological changes of the lung. The pulmonary hemorrhage and congestion occurred in the lung of Bartha-K61-, HB98- and DCD1-inoculated dogs. The lesions were labeled with arrows. Magnification, ×200.

autonomous regions of China) in diverse species, mainly in pigs but also in cattle, sheep, goats, cats, dogs and minks (Sun et al., 2016).

PRV has a double-stranded linear DNA genome with 150 kb in length, encoding at least 11 different glycoproteins (gB, gC, gD, gE, gG, gH, gI, gK, gL, gM and gN) (Dong et al., 2014). To data, only *gB gD*, *gH*, *gL* and *gK* were confirmed as the essential genes of PRV, whereas other genes are considered nonessential (Kopp et al., 2004; Olsen et al., 2006). The existence of these nonessential genes in the PRV genome permits the deletion or the insertion of foreign genes in the hope of vaccinating against PRV or other diseases (Hong et al., 2007). Thanks to this, many novel recombinant vaccines against PR have been developed in the past decades, such as the gE-gene-deleted PRV vaccine Bartha-K61, the gE/gI/TK-gene-deleted PRV vaccine SA215, the gG/TK-deleted PRV vaccine HB98, the gE/gI/TK-deleted PRV vaccine TJ, the gE/gI/TK-deleted PRV vaccine HN1201, and the killed gE/gI-deleted PRV vaccine ZJ01 (Cong et al., 2016; Gu et al., 2015; Zhang et al., 2015a). Although all these attenuated vaccines have been reported to provide effective protection against PRV infection, only Bartha-K61, HB98 and SA215 have been proved to be safe and efficacious, and licensed in China. The safety and efficacy of other candidate vaccines still need further evaluation.

Currently, all the licensed vaccines against PRV were widely used in Chinese swine industry, and played a critical role in control of this disease.

Bartha-K61 used in this study is an attenuated PRV strain which was deleted the gE and part of gI genes (Klupp et al., 1995). PR has been eradicated from domesticated pigs in North America and a number of European countries using the Bartha-K61 vaccine for large-scale compulsory vaccination and the differentiating infected from vaccinated animals strategy (Muller et al., 2011). The Bartha-K61 vaccine strain was imported from Hungary in 1979, and then widely used in Chinese swine industry. Currently, the PRV vaccines based on the Bartha-K61 strain or its derivatives were wide-scale immunized in the Chinese pig industry, resulting in relatively favorable control of PR and the morbidity and mortality in newborn piglets of infected swine herds were less than 10% in Bartha-K61-vaccinated swine herd (Sun et al., 2016). HB98 used in the present study is also an attenuated PRV strain, which was deleted the gG and TK genes. The vaccine based on the HB98 strain was licensed in 2006 and considered to be suitable for emergency vaccination in case of PR outbreaks. Nowadays, Bartha-K61 vaccine and HB98 vaccine are the most widely-used vaccines against PR in China, that is why we selected both strains in the present study.

Dogs have been reported to be the host of PRV, the pathogenesis of natural and experimental PRV infection in dogs has been evaluated. However, few reports about the pathogenesis of attenuated PRV vaccine strains in dogs are available. Therefore, we investigated the PRV seroprevalence and assessed the risk factors of PRV infection in dogs in the present study. Considering the PRV infection in dogs, we performed the epidemiological survey using the serum specimens that were collected form dogs in pig farms and pet hospitals from May 2017 to June 2018. Our data strongly suggested that PRV, especially the gE-deleted PRV strains, existed in dogs in South China. However, no PRV strains were isolated in this epidemiological survey because of the absence of clinical specimens. More effort will be required to determine the prevalence

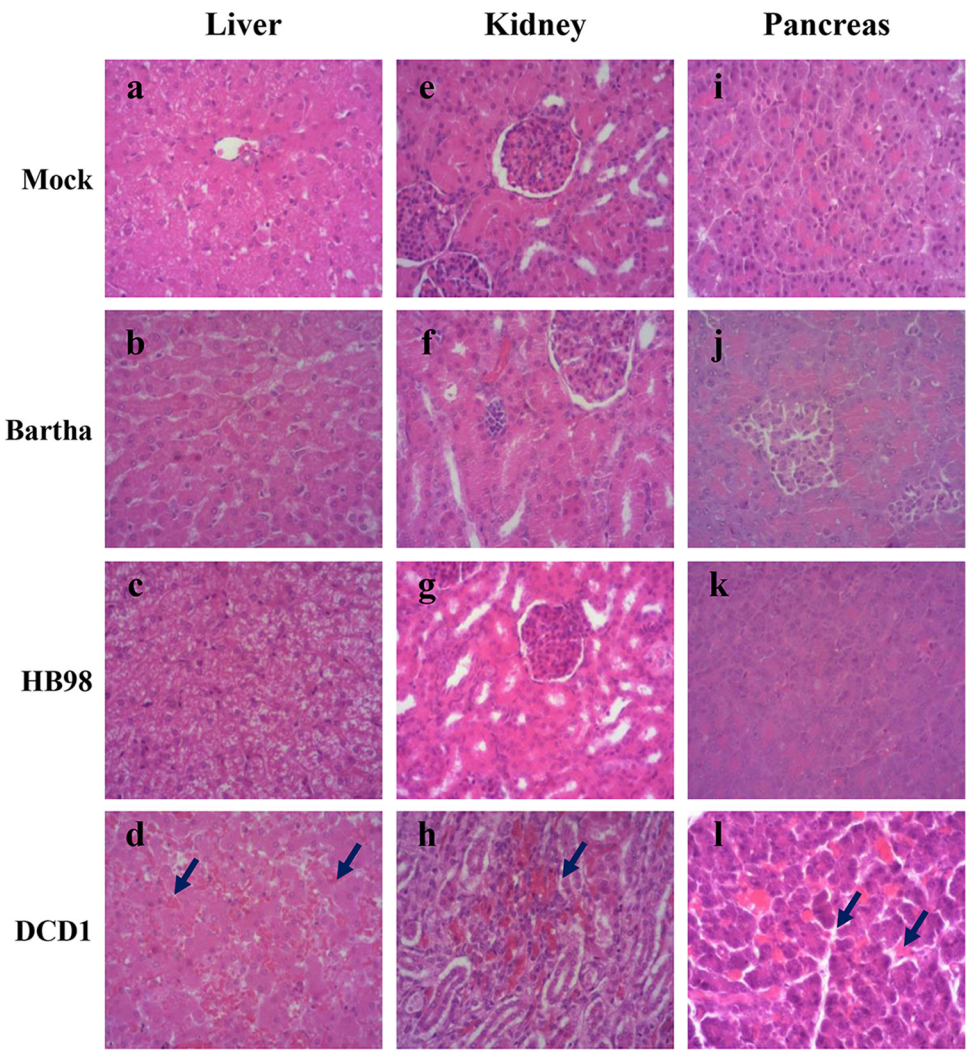

Fig. 4. Histological changes in dogs infected with PRV. Affected tissues of mock-, Bartha-K61-, HB98- and DCD1-infected dogs were fixed by immersion in 10% neutral-bufered formalin, routinely processed, embedded in parafn, sectioned (4 μm thick), and stained with hematoxylin and eosin. Histological changes were examined by light microscopy. (a–d) Histological changes of the liver. The hemorrhage occurred in the livers of DCD1-inoculated dogs, while no typical lesions were observed in other dogs. (e–h) Histological changes of the kidney. The hemorrhage occurred in the kidneys of DCD1-inoculated dogs, while no typical lesions were observed in other dogs. (i–l) Histological changes of the pancreas. The hemorrhage occurred in the pancreas of DCD1-inoculated dogs, while no changes were observed in the Bartha-K61- and HB98-infected dogs. The lesions were labeled with arrows. Magnification, ×200.

of PRV in dogs.

Of note is that our findings were obtained primarily from experiments using animals. According to the previous reports, the incubation time of PRV is about 2–9 days in dogs, and most infected dogs die within 48 h of the onset of symptoms, some dogs even die without showing any of the typical symptoms (Capua et al., 1997; Monroe, 1989), the typical symptoms caused by PRV are characterized by pruritus, dyspnea, vomiting, bloody diarrhea, edema, ataxia and muscle spasms in dogs (Cramer et al., 2011). Consistent with these reports, in our study, we found that both the virulent strain DCD1 and the attenuated strain HB98 cause similar clinical symptoms and pathological lesions, and induce death in 24 h of the onset of symptoms. The incubation time of DCD1 and HB98 were 2 days and 5 days, respectively. Interestingly, there is a discrepancy for the pathogenicity of Bartha-K61 in dogs. The early studies showed that the Bartha strain are dangerous for dogs, because the outbreak of this disease occurred after two week vaccination with Bartha strain in dogs (Willemse et al., 1977). Controversially, a recent report indicated that Bartha-K61 is not only safe for dogs, but also effective in inducing an immune response to the antigen it carries (Yuan et al., 2008). Surprisingly, in our study, Bartha-K61 did not cause any death of dogs during the experiment (14 days), the body weight curve of Bartha-K61-inoculated dogs demonstrated a consistently increasing trend line, and the temperature curves of Bartha-K61-inoculated dogs were demonstrated a relatively stable trend line, yielding the trend lines similar to that of the mock infected-dogs. It seems that Bartha-K61 has no pathogenicity to dogs. However, in a follow up transmission experiments with Bartha-K61 vaccine, the initially inoculated as well as one of the contact-exposed dogs succumbed during the prolonged observation period (28 days), all the dogs presented with focal ecchymoses in the lung and spleen, and multifocal hemorrhages in the stomach and kidney, illustrating the potential risk associated with vaccinating dogs with Bartha-K61 vaccine. Therefore, we proposed that, when dogs are infected with Batha-K61, the virus with longer incubation time and lower replicate rate than other PRV strains (HB98 and DCD-1 strain) need more time to cause lesions in dogs. Our findings provide clues that the commercial vaccine Bartha-K61 is not safe for dogs.

The immune system plays a key role in protecting the body from foreign pathogens through either innate immunity or acquired immunity (Biron, 1998; Trevejo et al., 2001; Zhao et al., 2018). The innate cytokines, such as IFN-β, TNF-α, IFN-γ and interleukin, play a critical role in inhibiting virus infections. Although the dogs have been confirmed to be infected with PRV, few reports about the immune response of dogs against PRV infection are available. Our findings indicated that all the PRV-infected dogs produced much more IFN-β than controls, illustrating the dominant role of IFN-β during the early phase of PRV infection. Moreover, we also observed the increase of TNF-α in the HB98- and DCD1-inoculated dogs during the infection period, but only observed the increase of TNF-α in Bartha-K61-inoculated dogs at 14 dpi. This finding is consistent with the observation about the temperature changes in Bartha-K61-inoculated dogs. We proposed that Bartha-K61 with long incubation time and low replicate rate induced

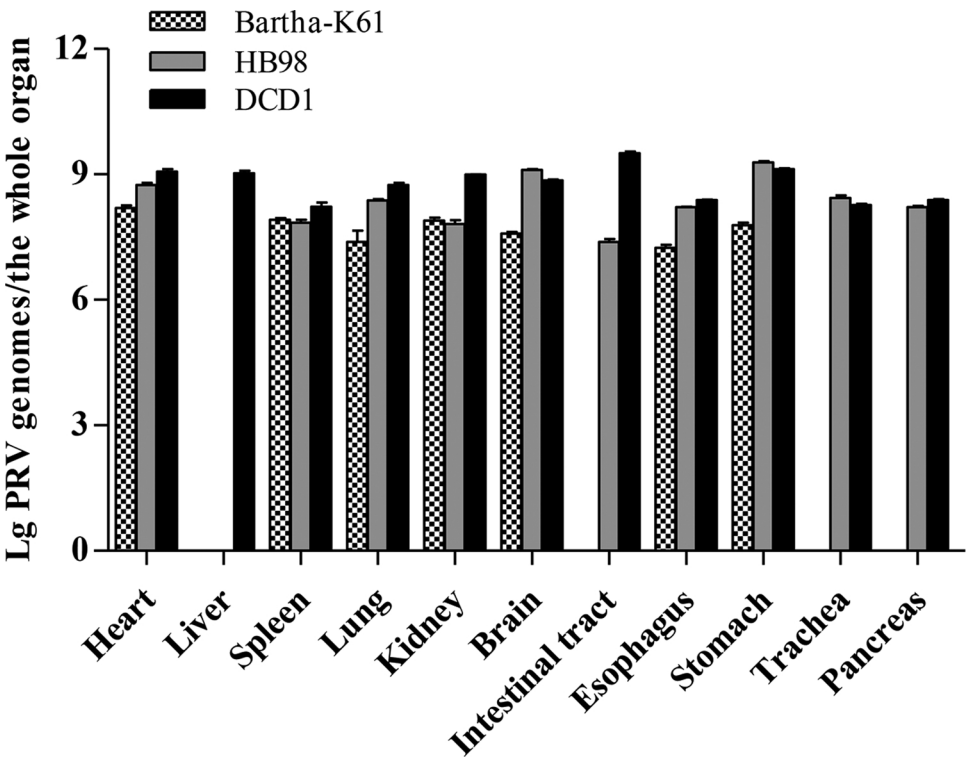

Fig. 5. PRV loads in the organs of artificially challenged dogs. 60-day-old beagle dogs were randomly divided into four group (n = 3), and inoculated with PRV strains Bartha-K61, HB98, DCD-1 or PBS as a control. All of the dogs were euthanized and subjected to analysis of viral loads/virus distribution of PRV. Data from Group IV (uninfected group) are not visible in the graph because there was no viral load in animals from that group. Results are representative of three independent experiments. Data are represented as means ± SD.

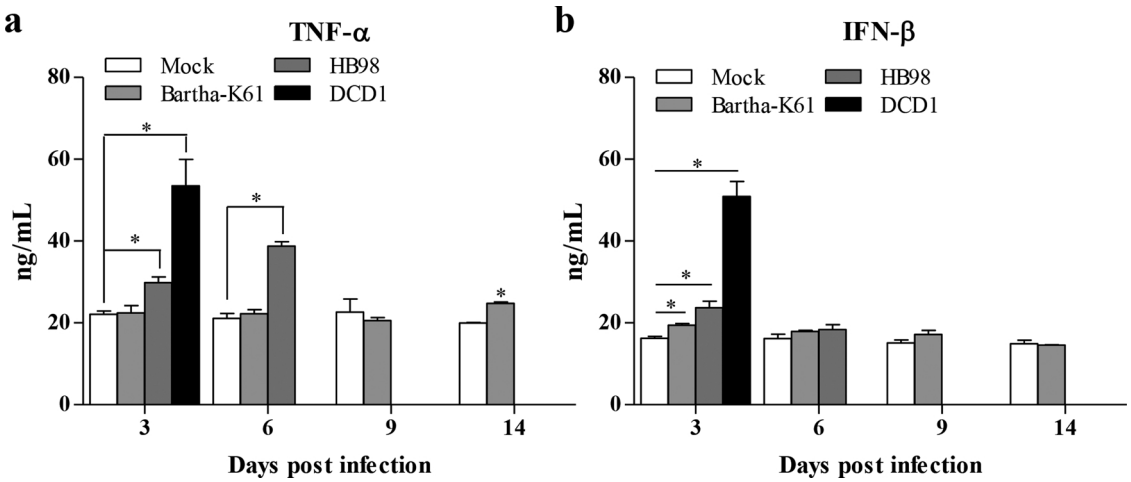

Fig. 6. Pro-inflammatory cytokines production in artificially challenged dogs. The blood samples were collected from the fore leg vein at 3, 6, 9 and 14 dpi, and processed to collect sera for detection of cytokines. The pro-inflammatory cytokines TNF-α (a) and IFN-β (b) in the serum were determined using ELISA Kit. Results are representative of three independent experiments. Error bars are presented as mean ± SD. The differences between the two groups are statistically significant as determined by two-way ANOVA (* stands for $p < 0.05$).

Table 3
Horizontal transmission of PRV between dogs.

Group	Animal Number	Endpoint	Shedding virus
Bartha-K61	I	Dead at 26 dpi	+
	II	Dead at 28 dpi	+
	III	Dead at 28 dpi	+
contact-expose	I	Dead at 28 dpi	+
	II	Euthanized	+
	III	Euthanized	+

the production of TNF-α, leading to the temperature increase from 12 dpi to 14 dpi.

Up to now, little reports about the viral distribution in the organs of the dogs are available. In the present study, we evaluated the viral loads of different PRV strains in the organs of the artificially challenged dogs. Interestingly, we detected the virus in all the organs of the DCD1-inoculated dogs, whereas no virus were detected in the liver of HB98- or Bartha-K61-inoculated dogs. Moreover, no virus were detected in the intestinal tract, trachea and pancreas of Bartha-K61-inoculated dogs. These results provide clues that Bartha-K61, HB98 and DCD1 have different replicate rate and tissue tropism in *vivo*. Considering the different deleted genes of Bartha-K61 and HB98, we proposed that the deleted genes might be associated with replicate rate and tissue tropism in *vivo*. Of course, further evidence is required to confirm this hypothesis. Additionally, Neuronal spread of PRV requires axonal sorting of assembled virions followed by transporting of viral particles over long distances in axons (Smith, 2012). The PRV Bartha strain with a 3.4 kb deletion (coding for gI, gE, US9 and US2) in the unique short

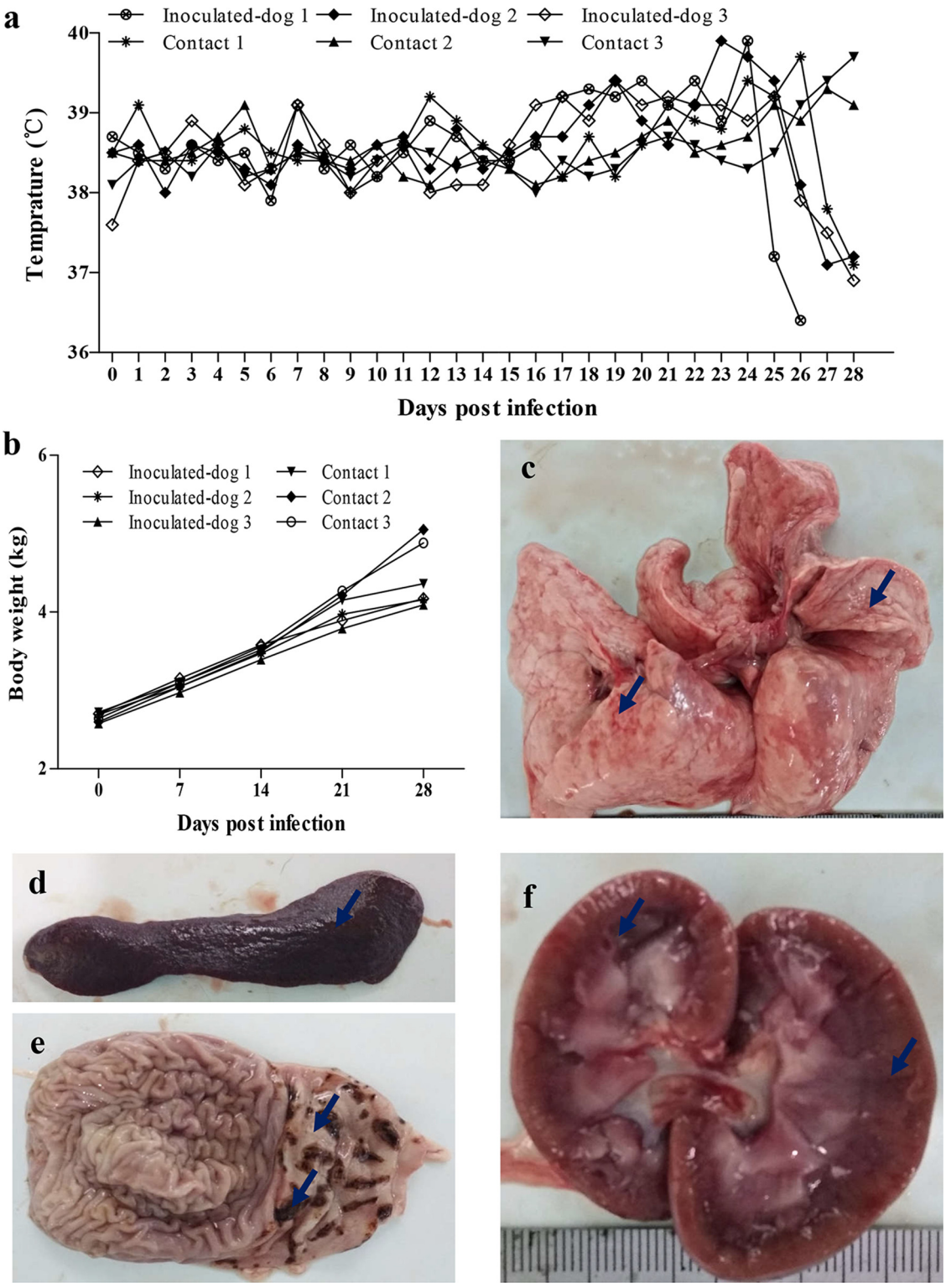

Fig. 7. Clinical features of Bartha-K61-inoculated dogs and contact-exposed dogs during the prolonged observation period. Three Bartha-K61-inoculated dogs were raised with three mock-inoculated dogs in the same room. Clinical signs of disease and mortality were monitored daily. All the dogs were euthanized till sudden death occurring. The autopsy was performed, and the gross abnormalities were recorded at necropsy. (a) Variation of rectal temperature in each dogs. (b) Body weight changes for each dogs. (c) focal ecchymoses were observed in the lung. (d) focal ecchymoses were observed in the spleen. (e) multifocal hemorrhages were observed in the stomach. (f) multifocal hemorrhages were observed in the kidney. The lesions were labeled with arrows.

region of the genome and some point mutations within the glycoprotein C, gM and UL21 genes, only spread from post- to pre-synaptic neurons in a circuit (retrograde only) (Paulus et al., 2006; Zeng et al., 2018). However, the effect of the impaired intraaxonal transport process on the replication, spread and tissue tropism of Bartha strain is unclear, more effort will be required to determine whether the effect exist.

It has been reported that dogs can be infected with PRV by consuming contaminated raw pork or by exposing to the virus during hunting or fighting with live pigs (Monroe, 1989; Quiroga et al., 1998), but very few reports about the transmission of PRV between dogs are

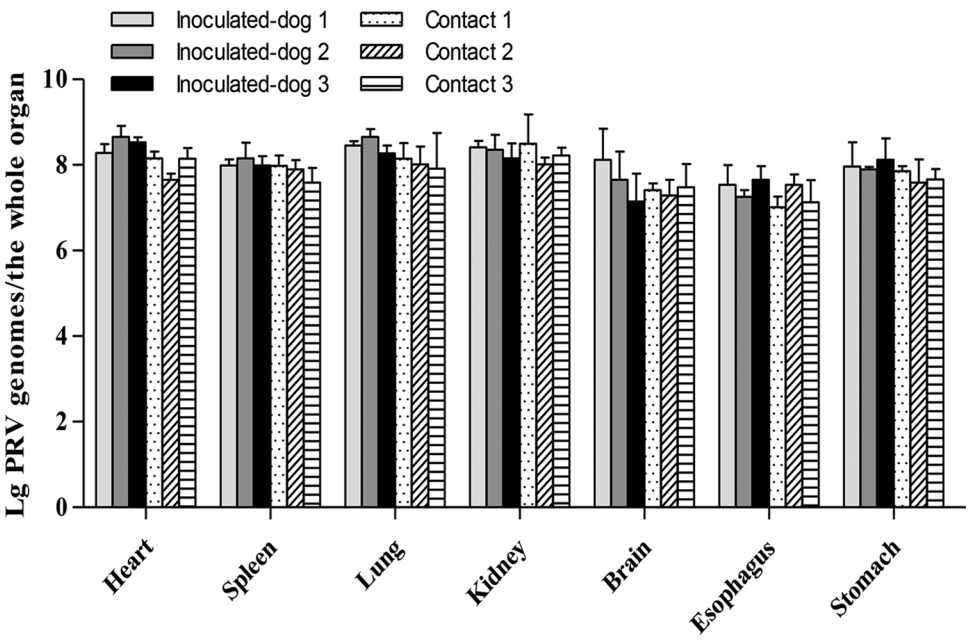

Fig. 8. PRV loads in the organs of Bartha-K61-inoculated dogs and contact-exposed dogs. Three Bartha-K61-inoculated dogs were raised with three mock-inoculated dogs in the same room. All of the dogs were euthanized and subjected to analysis of viral loads/virus distribution of PRV. Results are representative of three independent experiments. Data are represented as means ± SD.

available. Therefore, we attempted to elucidate the characteristics of PRV transmission in dogs. We first determined that contact-exposed dogs raised with Bartha-K61-inoculated dogs together can be infected with PRV, the same PRV strain was isolated in contact-exposed dogs. Moreover, our data also showed that Bartha-K61 cause death in dogs. These observations clearly show that PRV can be transmitted between dogs by touching with each other.

5. Conclusion

It is the first demonstration of the safety assessment of commercial attenuated PRV vaccines in dogs. Based on the epidemiological survey, pathogenicity evaluation and horizontal transmission in dogs, this study strongly suggest that commercial PRV vaccines are hidden health risk for dogs. Considering the susceptibility and the intimate relationship of human and pet dog, asymptomatic carriage of PRV in dogs at the early stage is also a hidden infection that it is vital for owner of pet dogs to take seriously.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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