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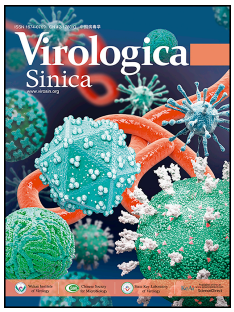

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Research Article

Insights into cross-species infection by coronavirus: Porcine epidemic diarrhea virus infections in the rodent

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Highlights:

1. The porcine epidemic diarrhea virus (PEDV) can cause systemic infections in neonate mice and rats.
2. PEDV has the cerebric tropism in piglets.
3. PEDV poses the potential to cross the species barrier and infect the rodent.

ABSTRACT

The cross-species infection of coronaviruses has resulted in several major epidemics since 2003. Therefore, it is of great importance to explore the host ranges of coronaviruses and their features among different hosts. In this study, the porcine epidemic diarrhea virus (PEDV), with swine as the only natural reservoir, was detected in rat fecal samples collected from pig farms. Further animal tests showed PEDV can cause systemic infections in neonate mice and rats. The brain, lung intestine and spleen were all targets for PEDV in rodents in contrast to the intestine being targeted in pigs. Morbidity and mortality vary via different infection routes. PEDV was also detectable in feces after infection, suggesting that the infected rodents were potential infectious sources. Moreover, the cerebric tropism of PEDV was verified in piglets, which had not been identified before. In conclusion, our findings demonstrate that PEDV can cross the species barrier to infect mice and rats through different routes. Although it is highly devastating to piglets, PEDV changes the target organs and turns to be milder when meeting with new hosts. Based on these findings, more attention should be paid to the cross-species infection of PEDV to avoid the emergence of another zoonosis.

Keywords: Porcine epidemic diarrhea virus; rodent; cerebric tropism; cross-species; animal model.

INTRODUCTION

Porcine epidemic diarrhea (PED) is a devastating enteric disease in pigs, characterized by severe enteritis, vomiting, and watery diarrhea. The causative agent, porcine epidemic diarrhea virus (PEDV), is an enveloped, single-stranded, positive-sense RNA virus belonging to the *Coronaviridae* family. PEDV can infect pigs of all ages, but morbidity and mortality vary. It can cause 100% mortality in piglets within two weeks but is much milder in pigs older than two weeks. Since its first discovery in the 1970s (Wood, 1977; Pensaert and de Bouck, 1978), PEDV has subsequently spread worldwide. In the 2010s, variant PEDV strains re-emerged, eliminating millions of suckling piglets (Sun RQ. et al., 2012; Stevenson et al., 2013; Chung et al., 2016; Sun Y. et al., 2019). To date, the virus is still evolving and circulating worldwide.

Pigs are identified as the only natural reservoir for PEDV. However, this virus exhibits poor replication in swine cells. Both IPEC-J2 and IPI-2I, two swine intestine cell lines, are permissive for PEDV infection but with limited viral replication (Zhao et al., 2014; Wang X. et al., 2019). In contrast, cell lines derived from other species are susceptive to PEDV. The rat crypt epithelial cell line (IEC-6), Vero cells, 293 cells, various human hepatocyte cell lines and human intestinal cell lines support PEDV replication to high titers (Zhang et al., 2017; Chen J. et al., 2020; Lv et al., 2022; Niu et al., 2023). Recently, several PEDV-like coronaviruses were also identified by virome analysis in bats in China, Laos and Brazil (Simas et al., 2015; Lacroix et al., 2017; Han et al., 2019). Although only partial genomic information was provided, these findings raise public concern about whether PEDV will be capable of jumping to non-porcine hosts or if it is already in the process of doing so.

In the 21st century, the outbreaks of severe acute respiratory syndrome coronavirus, Middle East respiratory syndrome coronavirus and severe acute respiratory syndrome coronavirus 2 are all related to the cross-species infection of coronaviruses. Therefore, it is important to investigate whether PEDV can infect rodents. In this study, PEDV was detected in rat fecal samples collected from pig farms. BALB/c mice and SD rats were used for further verification. The results

demonstrated that PEDV could cross the species barrier to infect the rodent, providing valuable insights into PEDV behavior.

RESULTS

The PEDV genome is detectable in rat fecal samples collected from pig farms

Infestations associated with rodents have been a persistent challenge for pig farms. Several porcine viruses have previously been isolated in rats (Tonietti Pde et al., 2013; Joshi et al., 2016; Zhai et al., 2016; Murphy et al., 2019; De Sabato et al., 2020). In a prior study, we identified that the rat crypt epithelial cell line, IEC-6, was highly susceptible to PEDV, suggesting rats may be potentially susceptible to PEDV (Chen J. et al., 2020). Consequently, we endeavored to further detect PEDV in rat fecal samples collected from pig farms during 2020 and 2022. Among the collected samples, 6 out of 39 were found to be positive for PEDV. Two complete PEDV genomes were then assembled (GenBank access No. OR601542 and No. OR601543). Phenotypic analysis based on the complete genome indicated that the obtained PEDV strains belong to the circulating GIIb cluster (**Fig. 1**). The information of other reference PEDV strains was listed in **Supplementary Table S1**. Although PEDV was detected in rat fecal samples, it is challenging to deduce whether rats were infected by PEDV or if the samples were contaminated by PEDV during collection. Therefore, our next step was to attempt to infect mice with PEDV.

Neonate mice are permissive to PEDV via different inoculation routes

To assess PEDV infection in mice, 7-day-old BALB/c mice were selected and inoculated with PEDV using various routes. Intraperitoneal (i.p.), intragastric administration (i.g.), oral, and intranasal (i.n.) inoculations were applied with 10^5 $TCID_{50}$ PEDV per mouse (**Fig. 2A–D**). Each group of the survival monitoring experiment contained five mice. In the pathology progression experiment, nine mice were sacrificed at 1, 3, and 5 days post-inoculation (dpi) for viral load determination.

The results showed that the i.p. mice and orally inoculated mice displayed no

significant clinical symptoms (**Fig. 2E and 2F**) One out of five mice with i.g. died at 5 dpi. The infected mice exhibited anorexia and depression, with watery diarrhea being observed at 2 and 3 dpi in some mice (**Fig. 2G**). Intranasal mice showed no deaths, but depression and soft feces were observed at 2 and 3 dpi and recovered thereafter (**Fig. 2H**). All infected mice maintained stable growth during the observation period, except for the i.g. group, which showed significantly slower growth compared to the mock group. (**Fig. 2I–L**).

At 1, 3, and 5 dpi, three mice from each group were euthanized. Different tissues were harvested for viral load determination and RT-PCR analysis. Surprisingly, PEDV was detected among different tissues. Mice in the i.p. (**Fig. 2M**) and had the highest viral titers in the brains and lungs, exceeding 10^2 $TCID_{50}$/mg tissue. PEDV was also detectable in the lungs, spleens, intestines, and blood for all mice at 3 dpi. In orally inoculated mice, PEDV was only detected in the brains and lungs at 3 dpi (**Fig. 2N**). Additionally, PEDV was detectable in the brains, lungs, intestines, and blood for i.g. and i.n. mice (**Fig. 2O and 2P**). These results were confirmed by RT-PCR analysis. The results revealed strong bands in the brains and lungs, faint bands in the spleens, intestines, or blood, but no signal in other tissues of all inoculated groups at 3 dpi (**Fig. 2Q**). All these data suggest that PEDV can establish infections in neonate BALB/c mice via different routes.

Neonate mice are susceptible to PEDV via intracranial inoculation

The preceding results suggest that PEDV can infect neonate mice at low levels and efficiently replicate in the brain. Therefore, our next step was attempting to infect mice with PEDV via intracranial inoculation (i.c.) (**Fig. 3A**). We infected 7-day-old mice with PEDV via i.c. (10^5 $TCID_{50}$ PEDV for each mouse). After inoculation, the PEDV-infected mice exhibited depression and anorexia at 1 dpi (**Fig. 3B**). Subsequently, it was observed that PEDV infection delayed the growth of infected mice compared to the control group (**Fig. 3C**). By 5 dpi, 3 out of 5 mice had died, while the remaining mice survived with their body weight increasing from 6 dpi (**Fig. 3D**). During the observation, the infected mice developed watery diarrhea, and their

body weight sharply dropped from 3 dpi (**Fig. 3E**). Some infected mice even developed opisthotonus. The intestine of infected mice was filled with yellow content and air, contrasting with white chyle-like contents in control mice (**Fig. 3F**). Although the size of the spleens and the length of the intestines were distinct between infected mice and control mice, there was no significant difference after dividing by body weight (**Supplementary Table S2**). The results indicated that PEDV was highly lethal to 7-day-old mice via i.c. Additionally, i.c. had nearly little influence on control mice (**Supplementary Fig. S1A and 1B**). Viral load determination showed that the brain was the most suitable for PEDV growth, with the top viral titers exceeding 10^4 $TCID_{50}$/mg. PEDV was detectable in the brain, lung, intestine, blood, heart, kidney and fece at 3 and 5 dpi (**Fig. 3G**). PEDV was also detectable in the spleens at 3 dpi and liver at 5 dpi, with viral titers much higher via i.c. than other inoculation routes. The data above demonstrated that PEDV displays cerebric tropism in neonate mice. More importantly, morbidity and mortality of PEDV in mice vary via different infection routes.

PEDV results in asymptomatic infection in mice older than 7 days

While PEDV can lead to a death rate of over 95% in suckling piglets, it manifests mild symptoms in pigs older than two weeks. To investigate whether PEDV infection in mice is also highly associated with age, 7-day-old, 14-day-old, and 21-day-old mice were used for further examination. The observation period was extended to 14 days for more detailed monitoring and collection serum for antibody response detection.

During the test, only inactivity, wasting, and depression were observed in 14- and 21-day-old mice (**Fig. 4A**). The body weight of 14- and 21-day-old mice slightly dropped after inoculation but recovered at 3 dpi (**Fig. 4B**). No mice older than 7 days died during the observation (**Fig. 4C**). The 7-day-old mice displayed severe diarrhea symptoms, and three out of five died at 3 dpi. The body weight of the surviving mice differed from that of the control mice. Viral load results revealed that PEDV infection mainly occurred in the brains of 14- and 21-day-old mice but with lower viral loads

compared to 7-day-old mice (**Fig. 4D–F**). PEDV was not detected in other organs for older mice, except in the lung of one 21-day-old mouse at 5 dpi. The viral titers in the brain also peaked at 3 dpi for all infected mice. At 14 dpi, PEDV was not detectable in the brains of 21-day-old mice. Additionally, seroconversion was detected in 7-day-old mice but not in older mice (**Fig. 4G–I**). In conclusion, PEDV also displayed age-related morbidity and mortality in mice via i.c.

The mortality of PEDV in mice is viral dose-dependent

To determine the lowest dose that can result in death, 7-day-old mice were inoculated with PEDV at different doses (10^5, 10^4, 10^3, 10^2, and 10^1 $TCID_{50}$) or DMEM. Each group contained three co-housed mice, which were also sacrificed at 14 dpi to assess the transmission of PEDV.

The results demonstrated that mice inoculated with 10^5, 10^4, and 10^3 $TCID_{50}$ PEDV displayed severe watery diarrhea (**Fig. 5A**). The death rates for these three groups were 60%, 40%, and 40%, respectively (**Fig. 5B**). All deaths occurred before 5 dpi. Although mice inoculated with 10^2 and 10^1 $TCID_{50}$ viruses appeared asymptomatic, one mouse inoculated with 10^2 $TCID_{50}$ viruses died at 11 dpi (**Fig. 5A and 5B**). The size and body weight of the mice inoculated with 10^2 or 10^1 $TCID_{50}$ viruses were quite like the control mice, and larger than other three groups (**Fig. 5C and 5D**).

The viral titration results showed that the replication of PEDV peaked at 3 dpi and decreased thereafter for all groups. At 14 dpi, PEDV could be detected in the brains, lungs, intestines, blood, and feces of mice inoculated with more than 10^3 $TCID_{50}$, suggesting a potential source of infection (**Fig. 5E and 5F**). For mice inoculated with 10^3 or 10^2 $TCID_{50}$ viruses, PEDV could be detected in the brains, lungs, intestines, and spleens before 7 dpi and occasionally in other tissues (**Fig. 5G and 5H**). For mice inoculated with 10^1 $TCID_{50}$ PEDV, the virus could only be detected in the brains and lungs before 7 dpi (**Fig. 5I**).

Seroconversion was then confirmed in inoculated mice, with OD_{450} nm values around 0.4–0.7, compared to about 0.2 for control mice (**Fig. 5J**). Although no virus

was detected in co-housed mice at 14 dpi, antibodies against PEDV-nucleocapsid protein were detectable in co-housed mice, except for mice co-housed with 10^1 and 10^2 $TCID_{50}$ inoculated mice (**Fig. 5K**) but not in the parent mice (**Fig. 5L**).

PEDV of different genotypes are fatal to neonate mice

PEDV has been categorized into two lineages, distinguished by differences in immunity and pathogenicity for piglets. The classic lineage, also known as genotype I, was initially identified in the 1970s and rarely detected since the 2010s, with the representative strain being CV777. The genotype II virus has been prevalent worldwide since 2013, and in the above studies, the genotype II LJX strain was used for examination. Here, the genotype I CV777 strain was also utilized to test for any differences.

7-day-old BALB/c mice were intracranially inoculated with 10^5 $TCID_{50}$ per mouse. The inoculated mice developed severe watery diarrhea at 1 dpi, and 3 out of 5 mice exhibited opisthotonus (**Fig. 6A and 6B**). Their body weight did not increase after inoculation (**Fig. 6C**), and all mice died within 5 dpi (**Fig. 6D**). Both the small and large intestines were filled with yellow content and air, while the large intestine turned transparent (**Fig. 6E**). Viral loading determination demonstrated the presence of the virus in various tissues at 3 and 5 dpi (**Fig. 6F**). The combination of high mortality and high viral loading in tissues suggests that PEDV from different genotypes is also destructive to neonate mice.

PEDV infection induces pathological changes in neonatal mice

The histopathological alterations in various tissues associated with PEDV infection were then determined in mice. The results revealed that PEDV infection in the brain led to pronounced neuronal edema. Furthermore, a satellite phenomenon was observed, wherein neurons were surrounded by oligodendrocytes, suggesting neuronal apoptosis. Additionally, the formation of cerebral thrombosis was significantly higher in infected mice (**Fig. 7A**). Control mice, on the other hand, showed no obvious lesions, implying no damage caused by intracranial inoculation. In

the lungs of infected mice, serious interstitial pneumonia was observed, characterized by thickening of the alveolar wall and achroacytosis. These findings suggest that PEDV also induces severe respiratory symptoms. Although the virus was detected in the intestine and spleen, only mild lesions were observed, with inclusion bodies accumulating primarily in the spleen (**Fig. 7A**). The immunohistochemistry analysis was performed to detect viral distribution using antibodies against the structural protein N or nonstructural protein 16 (**Fig. 7B**). PEDV was found to spread throughout the brain, with a high concentration in the cerebral cortex and fewer signals in the fornix. In the lung, strong signals were observed in the trachea and alveolar wall, indicating PEDV infection in the respiratory tract. Moreover, PEDV infected the intestine and spleen, with signals detected in the intestinal villus and medulla of the spleen. In conclusion, PEDV can result in systemic infection in mice, causing severe lesions in the brain and lung but milder effects in other tissues.

PEDV results in asymptomatic infection in sucking rats

As the mouse is identified to be susceptive to PEDV, we then test whether PEDV can infect the rat. Similar, 7-day-old rats were inoculated with 10^5 $TCID_{50}$ PEDV via i.p., i.g., oral, i.n. and i.c. All infected rats were alive and displayed no observable clinical symptoms during the observation for 7 days, which was distinct to mice. The viral titration assays showed that PEDV was also detectable among the brains, lungs, and intestines for each inoculation route before 5 dpi (**Fig. 8A–E**). PEDV was also detectable within the spleens, intestines and feces of rats derived from oral, i.g. and i.c. group at 7 dpi. The viral titers in rat intestines were higher than that in mouse intestines, suggesting rats may be susceptible to natural infection of PEDV via fecal-oral transmission than mice. Immunohistochemistry analysis also supported the results above. The positive signals were observed within the brains, lungs, intestines, and spleen. Although the signals within the rat brains and lungs were significantly reduced comparing to mouse, they were enhanced in the intestines and spleens, potentially explaining why PEDV was not lethal to rats (**Fig. 8F**).

PEDV infection is confirmed in the brains of piglets

While this study has identified that PEDV targets the brains of the rodent, this manifestation has not been verified in pigs. To gain a deeper understanding of PEDV infection, we selected three 5-day-old piglets and infected them with the PEDV LJX strain. All pigs developed severe clinical symptoms and succumbed to the infection by 4 dpi. The real-time PCR analysis revealed over 10^8 copies/mg of the PEDV genome in the intestine, whereas a relatively low number of PEDV genome copies were detected in pig brains and other tissues (**Fig. 9A**). The PEDV genome copies within the brains and other tissues closely approached the cut-off line. However, immunohistochemical staining demonstrated the presence of PEDV in the brain, identified among the neurons, leading to their necrosis in all three piglets, while positive signals were not observed in mock-infected pigs (**Fig. 9B**). Finally, Vero cells were infected with the filtered brain homogenate and analyzed with IFA. The presence of positive signals demonstrated the existence of infectious PEDV virions within the pig brain (**Fig. 9C**).

DISCUSSION

PEDV is an enteric virus belonging to the genus Alphacoronavirus within the *Coronaviridae* family. This virus infects pigs of all ages, causing mortality rates of up to 100% in suckling piglets. Since its first identification in the 1970s, PEDV has spread worldwide. Previous studies have established that pigs are the only identified natural reservoir for PEDV. However, in this study, we detected the PEDV genome in rat fecal samples and further discovered that mice and rats could be infected by PEDV via different routes. With variations in inoculation routes and doses, PEDV can result in either asymptomatic or fatal infections. Notably, PEDV infection in rodents differs from its manifestation in piglets, targeting the brains, lungs, and spleens. These findings indicate that rodents may be susceptible to PEDV.

Most coronaviruses exhibit a limited host range. However, those with the capacity for cross-species transmission can lead to disasters in both humans and

animals. Therefore, it is crucial to investigate the transmission of coronaviruses among different species. Mice are commonly used as an animal model for pathogenic and transmission evaluations. Several coronaviruses, traditionally with limited hosts, have been proven to replicate in mice, including HCoV-OC43, swine acute diarrhea syndrome coronavirus (SADS-CoV), porcine hemagglutinating encephalomyelitis virus (PHEV), and even the avian coronavirus infectious bronchitis virus (IBV) (McIntosh et al., 1967; Butler et al., 2006; Mora-Díaz et al., 2021; Chen Y. et al., 2022). However, the clinical symptoms observed in mice differ significantly from those in the natural reservoirs, often involving severe neurological signs and high viral titers in the central nervous system. These studies suggest that coronaviruses may change when encountering new hosts, evading detection.

Neonate mice have been experimentally infected with PEDV via intracranial inoculation in previous studies, yielding low morbidity and mortality in mice aged three days or younger (Kotani et al., 2013). However, the details of the infection were poorly characterized, with PEDV only detectable in the brain and not in other tissues. Moreover, transgenic mouse expressing porcine aminopeptidase N was also developed and proved to be susceptible to PEDV (Park et al., 2015). This study shows that PEDV can infect both mice and rats through various routes, leading to a range of manifestations from asymptomatic to fatal infections.

Our study also identified PEDV infection in pig brains in the comparative test. The viral titers were relatively low in the brain compared to the intestine, but infectious virions were present, causing neuronal necrosis. This manifestation has not been observed before and may potentially explain why the production capacity of PEDV-infected sows is unstable. It is also intriguing to note another swine coronavirus, PHEV, which primarily causes neurological signs but occasionally diarrhea for some variants (Mora-Díaz et al., 2019). Could PEDV occasionally switch its characteristics in some variants? PEDV variants, with a large deletion among the spikes, could infect both the lungs and intestines, which were observed previously. Similar mutations and results were also observed in TGEV. A large deletion within the TGEV spike also switched its tissue tropism to infect and replicate in the lung. It is

predictable that the PEDV variants will bring more uncertainty in the future.

Several swine viruses have been identified and isolated in rodents on pig farms. Porcine circovirus type 2 (PCV2), an economically important swine pathogen, has been reported to frequently spillover from pigs to rats on pig farms (Lorincz et al., 2010; Pinheiro et al., 2013; Zhai et al., 2016). Senecavirus A (SVA), an emerging picornavirus with swine as natural reservoirs, has also been detected and isolated from rat feces and small intestines collected from pig farms in the USA (Joshi et al., 2016). Additionally, rats captured from pig farms carrying the Hepatitis E virus (HEV) have drawn increasing attention (Sanford et al., 2012; De Sabato et al., 2020; Wang B. and Meng, 2021). Although pigs and wild boars were previously considered the only natural reservoirs for PCV2, the high positive rate of HEV in rats on pig farms complicates its control and prevention. Rodents seem to be a potential source for spilling porcine viruses to other species. Importantly, these viruses cause serious clinical symptoms in pigs but are often asymptomatic in rats. Therefore, their infection in rats is frequently overlooked, and the pathogenicity and transmission of these viruses in rats are poorly defined. Similar to these viruses, our study identified that PEDV infection turned out to be asymptomatic to rats. Although the replication was observed, no rats died via different infection routes, suggesting why PEDV may be ignored in rats.

The differences in immune responses and physiological conditions between the rodents and pigs might influence the phenotypes upon PEDV infection. Both sucking mice and rats have undeveloped blood-brain barriers, which may facilitate the entry and spread of PEDV. Pigs have more complex barriers between different tissues. Additionally, sucking pigs have better developed immune systems when compared to the rodents, which may further restrict the growth and spread of PEDV. These differences will primarily contribute to the distinct infection results between the rodents and pigs.

CONCLUSIONS

To our knowledge, this is the first study to provide robust data supporting the

potential cross-species infection of PEDV. With the neonate established as animal models, we found that PEDV could result in systemic infection in mice and rats. Morbidity and mortality are highly affected by inoculation routes and doses. Although efficient transmission was not observed, different strains of PEDV have acquired the ability to replicate in mice. More attention should be paid to the evolution and transmission of PEDV to prevent potential zoonosis.

MATERIALS AND METHODS

Virus and cells

Vero-E6 cells were purchased from the China National collection of authenticated cell cultures and maintained in our lab. The cells were cultured with DMEM (Sigma, Germany) supplemented with 10% fetal bovine serum (Sigma, Germany) and 100 IU of penicillin per mL at 37 °C in the presence of 5% CO_2. The PEDV LJX01/GS/2014 and CV777 strains used in this study were propagated in our laboratory. LJX01/GS/2014, belonging to gene type II, shared nucleartide homology of 99.9% and 97.9% with the identified two strains. The CV777 strain was a representative strain of gene type I viruses, significantly different from the identified strains.

Sample collection and detection

The rat fecal samples were collected from family pig farms between 2020 and 2022. All samples were collected from different areas of the family pig farms but away from the pig house to reduce the potential contaminants. Only fresh rat fecal sample free of visible contaminants were collected. After collection, the samples were dissolved in sterile PBS and then detected by RT-PCR analysis.

Rodent infection experiments

The BALB/c mice and SD rats of 7-day-old were purchased from the Experimental Animal Research Center of Lanzhou Veterinary Research Institute (LVRI). Animal experiments followed the recommendations of the Guide for the Care

and Use of Laboratory Animals of LVRI. All rodents used in this study were detected to be free of PEDV, Mouse hepatitis virus and Murine Rotavirus and kept in the Experimental Animal Research Center of LVRI, which could ensure the absence of exogenous pathogens. The experiments included two independent sections: a survival monitoring experiment and a pathology progression experiment. The former included five mice or rats for each group. The latter included fifteen mice each group for a 7-day test and twenty mice each group for a 14-day test. And 10 rats for each group were used in the viral titer determination. To evaluate PEDV infection, rodents were inoculated via intraperitoneal (i.p.), intragastric administration (i.g.), oral, intranasal (i.n.), or intracranial (i.c.) routes with a 50 μL volume for each one. The control animals were inoculated with an equal volume of DMEM. All animals were observed daily for clinical signs, and survival. The severity of clinical symptoms was scored on a scale of five grades (see **Supplementary Table S3**).

Pig infection experiment

Four 5-day-old Landrace piglets, detecting to be free of PEDV, Transmissible Gastroenteritis Virus (TGEV), Porcine deltacoronavirus (PDCoV), African swine fever virus (ASFV) and Porcine Reproductive and Respiratory syndrome virus (PRRSV), were purchased from a pig farm and kept in the Experimental Animal Research Center of LVRI, which could ensure the absence of exogenous pathogens. Three pigs were orally inoculated with 10^6 $TCID_{50}$ PEDV LJX strain, while one served as negative control, inoculated with an equal volume of DMEM. Infected piglets died at 4 dpi, and the control was then euthanized. All tissues were harvested for viral loading determination. Brains were also prepared for immunohistochemical staining.

Viral load determination

For viral load determination, rodents from each group were euthanized by CO_2 at 1, 3, 5, 7, and 14 dpi. The blood was collected by cutting tails of the tested mice before euthanasia. The tissues, were weighed, and mixed with 1 mL PBS in ice

412 waiting for homogenation within 15 min. The homogenates were then centrifuged at
413 12 000 rpm for 10 min at 4 ℃, and filtered with 0.45 μm filters. The filtrate was
414 serially diluted (10^{-1} to 10^{-6}) and inoculated in Vero-E6 cells. After 1 h post-infection,
415 cells were washed three times with PBS and incubated for a further 72 h. Viral titers
416 were checked by immunofluorescence assay (IFA) with a fluorescence microscope
417 (TE2000U; Nikon) and calculated by the Reed-Muench method.
418
419 **Immunofluorescence assay**
420 Vero-E6 cells were seeded in 96-well plates. At 85% confluency, cells were
421 infected with filtered tissue homogenate. After 48 hours, cells were treated with 4%
422 paraformaldehyde for fixing, 0.5% Triton X-100 for permeation, and 5% skim milk
423 for blocking. Incubation with mouse anti-PEDV-nucleocapsid monoclonal antibody
424 (1:5000 dilution) and DyLight 488 goat anti-mouse IgG antibody (1:1000 dilution)
425 (Biodragon, China) followed. Cells were examined under a fluorescence microscope
426 (TE2000U; Nikon) with a video documentation system.
427
428 **Antibody response against PEDV infection**
429 Serum collected from PEDV-infected mice, co-housed mice, parent mice, and
430 control mice at 14 dpi was used for ELISA. The serum (1:50 dilution) was incubated
431 with an ELISA plate coated with PEDV-nucleocapsid protein (100 ng per well) at
432 37℃ for 1 hour. After three washes with PBS, HRP-conjugated goat anti-mouse IgG
433 (H+L) (1:500 dilution) was added, and absorbance values were scanned at OD_{450} nm
434 with a microplate reader.
435
436 **RT-PCR and real-time PCR analysis**
437 RT-PCR analysis for PEDV detection used 2X TsingKe Master Mix (TsingKe,
438 China). Reactions were incubated at 95 ℃ for 3 min and subjected to 35 cycles of
439 95 ℃ for 15 s, 52 ℃ for 15 s, and 72 ℃ for 30 s, with a final extension step of 72 ℃
440 for 5 min. PCR products were analyzed by 1% agarose gel electrophoresis. Real-time
441 qPCR for PEDV genome copies detection used Universal U+ Probe Master Mix V2

(Vazyme, China) by the Bio-Rad CFX96 system, which was described in previous study (Huang et al., 2019). Reactions were incubated at 94 ℃ for 30 s, followed by 45 cycles at 94 ℃ for 5 s and 60 ℃ for 30 s.

Histopathology and immunohistochemical staining

The rodents infected with PEDV or uninfected were euthanized by CO_2 at 3 dpi for histopathologic and immunohistochemical examination. Tissues were immediately harvested, fixed in 10% formalin at room temperature for at least 48 h, bisected, and embedded in paraffin. Sections were deparaffinized, rehydrated by xylene three times, and dehydrated by ethanol of different concentrations. For histopathologic examination, tissue sections were stained with hematoxylin and eosin. For immunohistochemical testing, tissue sections were dewaxed and dehydrated. After deparaffination, sections were treated with 3% H_2O_2 for 10 min, followed by distilled water washing and microwave treatment in a citrate buffer for 20 min at 99 ℃. Sections were then blocked with 5% BSA for 30 min and further incubated with mouse anti-PEDV-nucleocapsid antibodies (1:1000 dilution) or rabbit anti-PEDV-nsp16 antibodies (1:500 dilution) for 1 h at 37 ℃, followed by biotinylated goat anti-mouse antibodies (1:500 dilution) or goat anti-rabbit antibodies (1:500 dilution) (Abbkine, China) for 30 min at room temperature. Sections were treated by 3, 3-diaminobenzidine tetrahydrochloride chromogen (Beyotime, China), counterstained with hematoxylin and visualized by a light microscope.

Statistical analysis

For statistical significance examination, the Student's *t*-test was used between matched groups. An unadjusted *P* value of less than 0.05 was considered significant, and a *P* value of less than 0.01 was considered extremely significant.

DATA AVAILABILITY

All the data generated during the current study are included in the manuscript.

ETHICS STATEMENT

The animal experiment was conducted in compliance with the Animal Ethics Procedures and Guidelines of Laboratory Animals, approved by the Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute (LVRI) of the Chinese Academy of Agriculturalcultural Sciences (Permit No. LVRIAEC-2022-076).

AUTHOR CONTRIBUTIONS

Jianing Chen: data curation, writing-original draft, project administration. Zemei Wang, Shengyu Lin, Yongheng Shao, Shuxian Li, Qingbo Chen, Yonghao Hu: data curation. Guangliang Liu: project administration, writing-review & editing.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.####

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588 **Figure legends**

589 **Fig. 1.** Detection and analysis of PEDV genome in rat fecal samples. The complete genome
590 sequences of two PEDV strains were successfully assembled and subjected to phylogenetic
591 analyses. The phylogenetic tree, constructed using the maximum likelihood (ML) method
592 with 1,000 bootstrap replicates, is presented. The red arch highlights the assembled PEDV
593 genome from this study.

594

595 **Fig. 2.** Neonate BALB/c mice are susceptible to PEDV infection via various routes. In this
596 study, neonate BALB/c mice were subjected to PEDV inoculation through different routes: (**A**)
597 intraperitoneal infection, (**B)** oral infection, (**C)** intragastric infection, and (**D**) intranasal
598 infection. Following inoculation, we monitored the challenged mice's clinical scores (**E, F, G,**
599 **H**), and body weight changes (**I, J, K, L**) daily for 7 days. Clinical scores were graded as
600 described in **Supplementary Table S3**. Subsequently, we homogenized the tissues of infected
601 mice for viral load determination and virus re-isolation. Viral load determination was
602 performed through $TCID_{50}$ detection (**M, N, O, P**) and RT-PCR analysis (**Q**).

603

604 **Fig. 3.** PEDV induces lethal outcomes in neonate BALB/c mice via intracranial inoculation.
605 **A** Seven-day-old BALB/c mice were intracerebrally inoculated with either PEDV or DMEM.
606 The daily observations included (**B)** clinical scores, changes in (**C)** body weight, and (**D)**
607 survival rates. **E** Post-inoculation, PEDV-challenged mice exhibited severe clinical symptoms,
608 particularly watery diarrhea. **F** The intestines of infected mice displayed distension with air
609 and fluidic, containing yellow content, while control mice had undigested, curdled milk
610 masses. **G** The viral load of PEDV in different tissues was determined by $TCID_{50}$.

611

612 **Fig. 4.** Age-dependent pathogenicity of PEDV in BALB/c mice. BALB/c mice of varying
613 ages were intracerebrally inoculated with either PEDV or DMEM. Daily observations
614 included (**A)** clinical scores, (**B)** changes in body weight, and (**C)** survival rates.
615 Post-inoculation, PEDV-challenged mice were euthanized, and tissues were harvested at
616 different days post-inoculation (dpi). The viral load of PEDV in different tissues was
617 determined by $TCID_{50}$ for (**D)** 7-day-old, (**E)** 14-day-old, and (**F)** 21-day-old mice.

Additionally, serum samples were collected from (**G)** 7-day-old, (**H)** 14-day-old, and **(I)** 21-day-old mice for detecting PEDV-specific IgG at 14 dpi.

Fig. 5. Dose-related morbidity and mortality in BALB/c mice upon intracerebral inoculation. 7-day-old BALB/c mice were intracerebrally inoculated with varying doses of PEDV or DMEM. Daily observations included (**A**) clinical scores, (**B**) survival rates, and (**C**) changes in body weight. **D** At 14 dpi, the size and condition of surviving mice varied among different groups. Post-inoculation, PEDV-challenged mice were euthanized, and tissues were harvested at 1, 3, 5, 7, and 14 dpi. The viral load of PEDV in different tissues for mice inoculated with (**E)** 10^5 $TCID_{50}$, **(F)** 10^4 $TCID_{50}$, **(G)** 10^3 $TCID_{50}$, (**H)** 10^2 $TCID_{50}$, and (**I)** 10^1 $TCID_{50}$ was determined. Additionally, serum samples were collected from (**J**) PEDV-infected mice, (**K**) co-housed mice and (**L**) parent mice for detecting PEDV-specific IgG at 14 dpi.

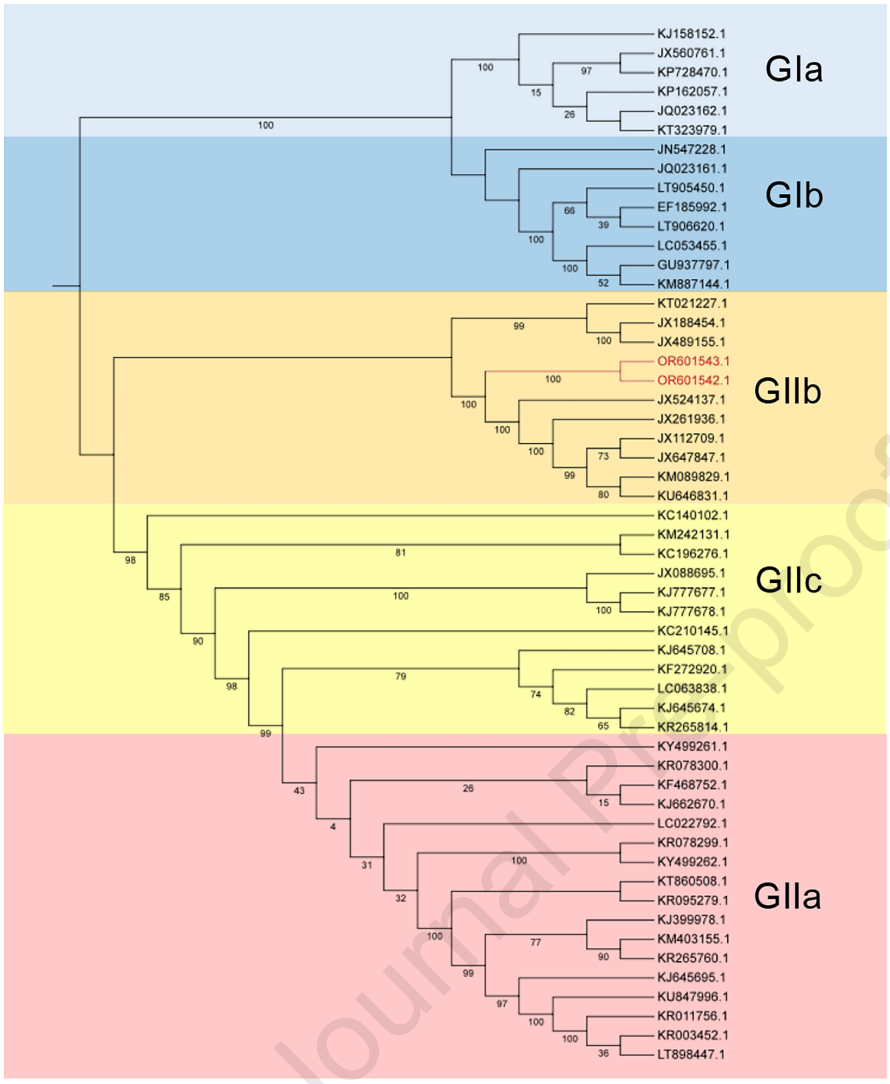
Fig. 6. Genotype I PEDV exhibits enhanced-pathogenicity in neonate mice. Seven-day-old BALB/c mice were intracerebrally inoculated with PEDV CV777 strain or DMEM. **A** Post-inoculation, CV777-challenged mice exhibited severe clinical symptoms from 1 dpi. Daily observations included (**B**) clinical scores, (**C**) changes in body weight, and (**D**) survival rates. **E** The small intestine of infected mice showed signs of air and fluid distension with a transparent appearance in the large intestine. **F** Viral titers of CV777 in different tissues were determined through $TCID_{50}$ analysis.

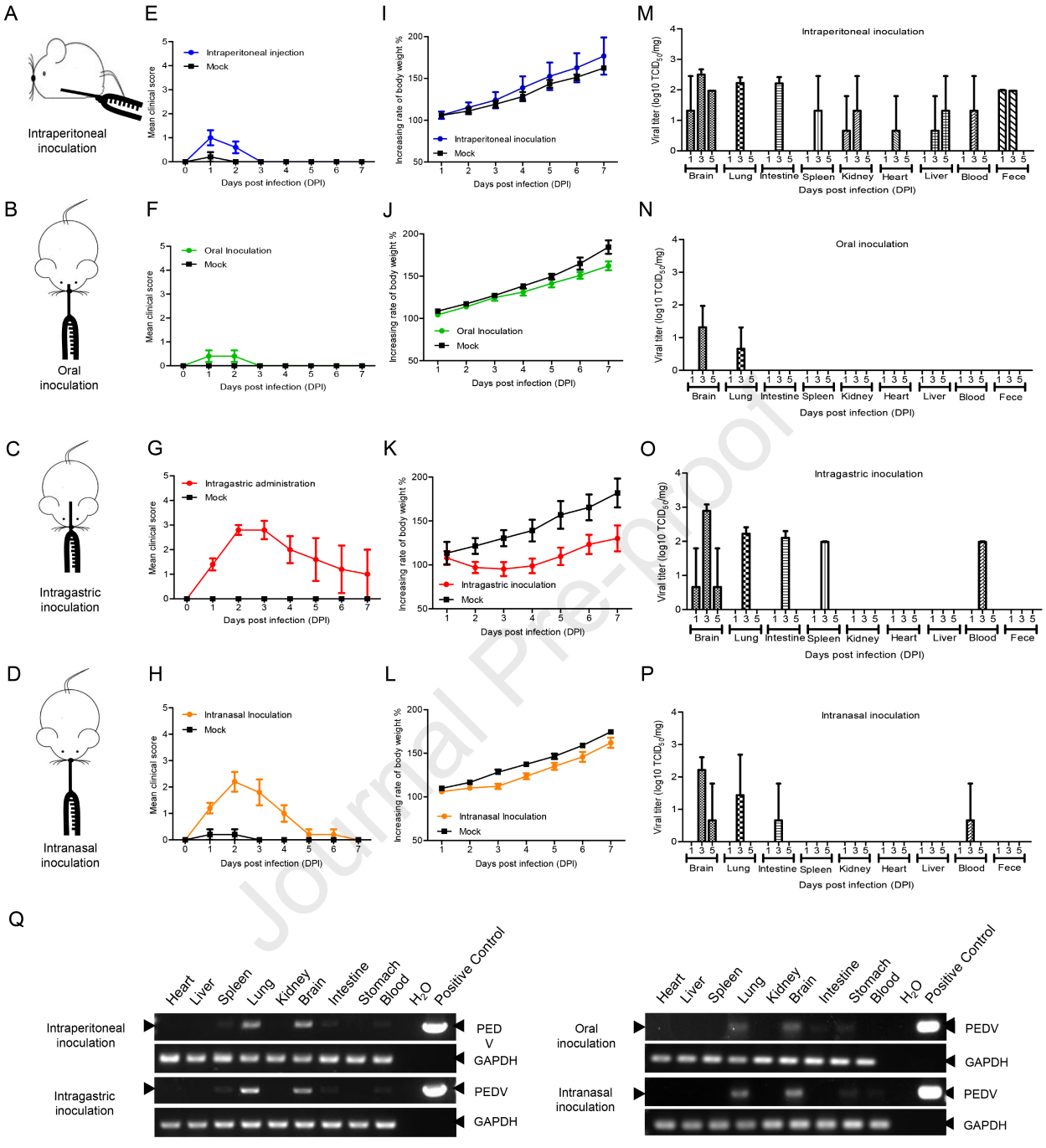
Fig 7. Pathogenic analysis of PEDV-infected mice. Seven-day-old BALB/c mice were intracerebrally inoculated with PEDV or DMEM and sacrificed at 3 dpi. The brain, lung, intestine, and spleen were collected and subjected to (**A**) H&E staining or (**B**) immunohistochemistry. Edema in the brain and lymphocyte infiltration in the lung were indicated by yellow arrows. The satellite phenomenon observed in the brain and the thickening of interstitial tissue in the lung were highlighted by red arrows. Thrombus formations were marked by black arrows, and inclusion bodies in the spleen were pointed out by red arrows. PEDV particles in different tissues were identified by red arrows in the immunohistochemistry images.

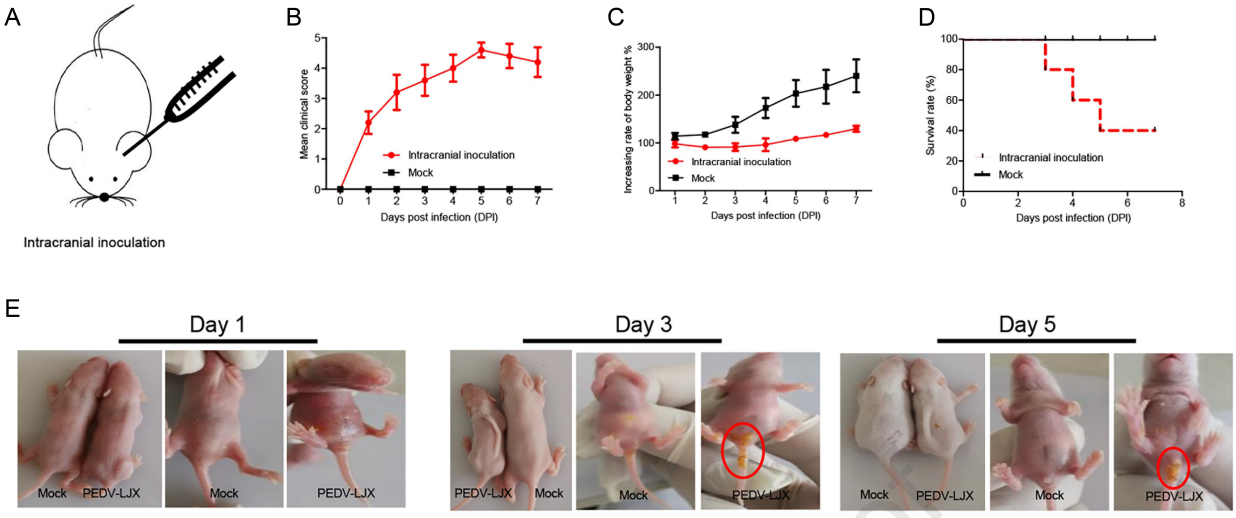
Fig 8. PEDV infection in 7-day-old rats. 7-day-old SD rats were inoculated with PEDV or DMEM via different routes for survival test or viral titer determination: (**A)** intraperitoneal infection, (**B)** intragastric infection, **(C)** oral infection, (**D)** intranasal infection and (**E**) intracranial infection. **F** Although no obvious lesions were observed, immunohistochemistry straining showed the presence of PEDV particles in different tissues, which were indicated by red arrows.

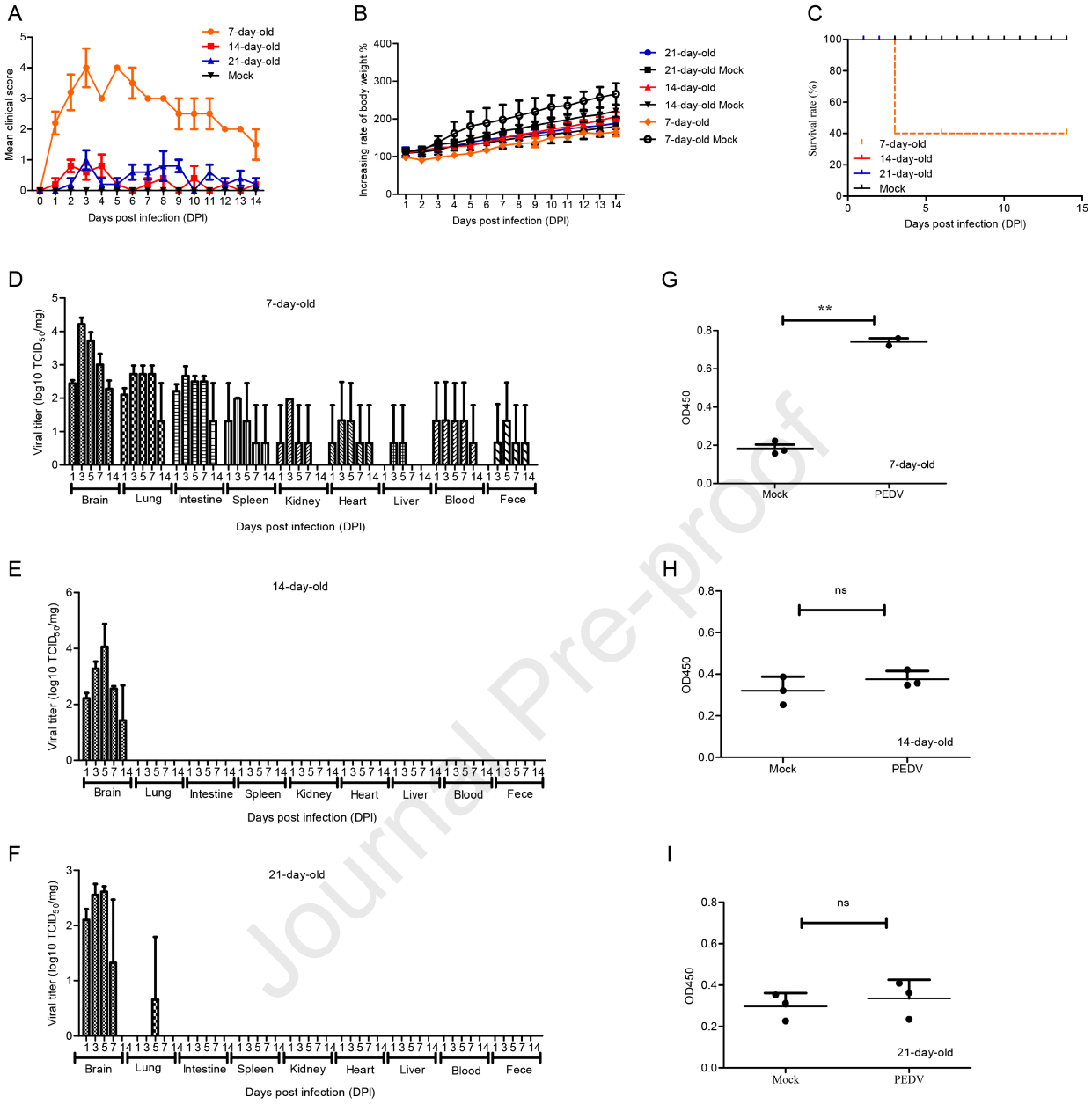
Fig. 9. Identification of PEDV infection in the brains of piglets. Three 5-day-old piglets were orally inoculated with PEDV, resulting in mortality by 4 dpi. One piglet was inoculated with an equal volume of DMEM as a control. **A** Various tissues, including the brain, were harvested for viral load determination using real-time PCR analysis. **B** Additionally, the brains were subjected to immunohistochemical staining to identify positive signals (red arrow). **C** Vero cells were inoculated with the filtered brain homogenate and analyzed using IFA to confirm the presence of infectious PEDV virions.

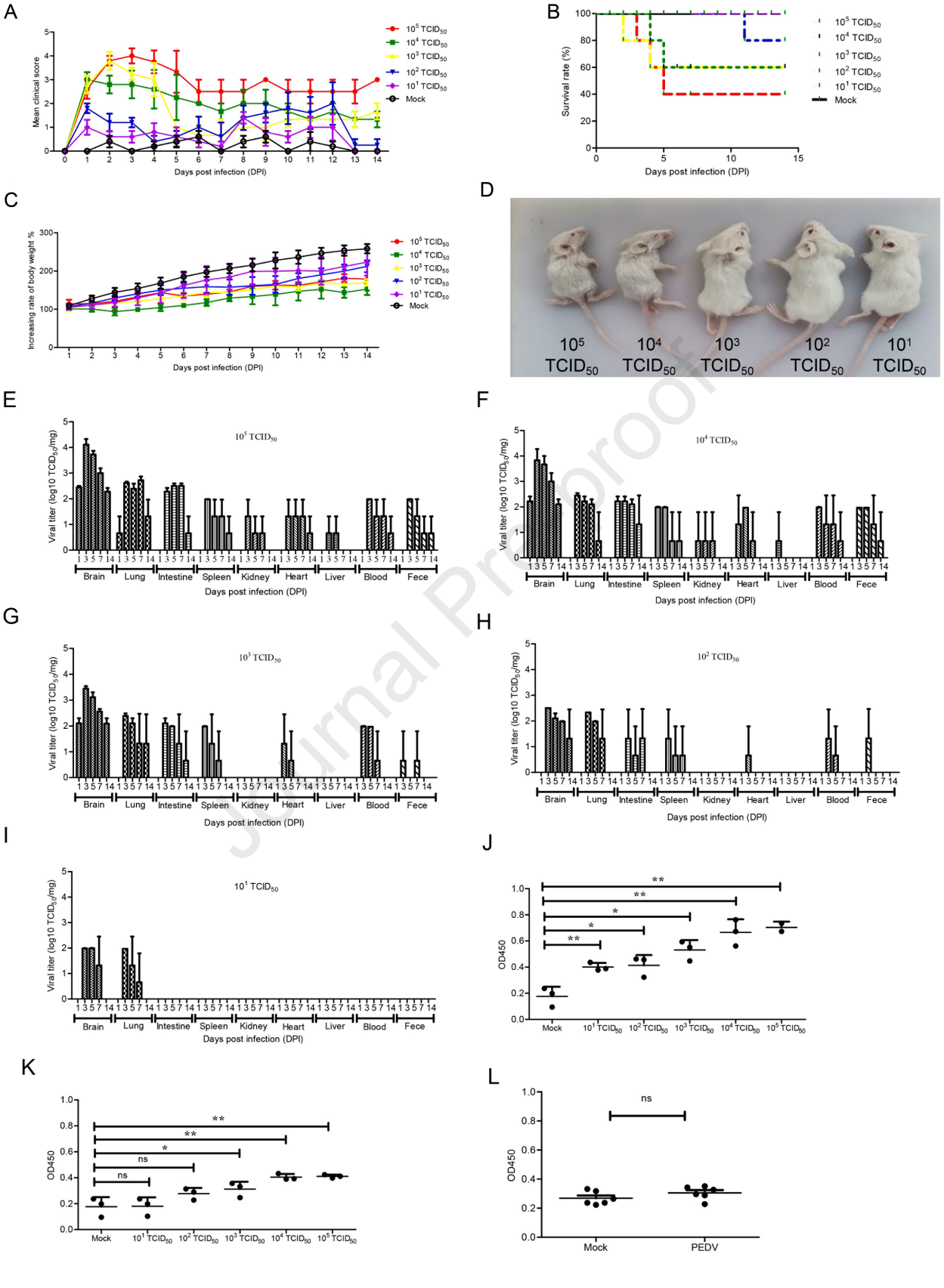
Supplementary Figure S1. Intracerebral inoculation causes minimal impact on suckling mice. Intracerebral inoculation (I.C.) did not induce vasoconstriction in challenged mice when compared to mice that were not treated (NT) (**A**). Furthermore, the mice subjected to intracranial inoculation exhibited rapid recovery, with wound healing observed within 24 hours (**B**). The arrows in (**B**) indicate the observed wounds.

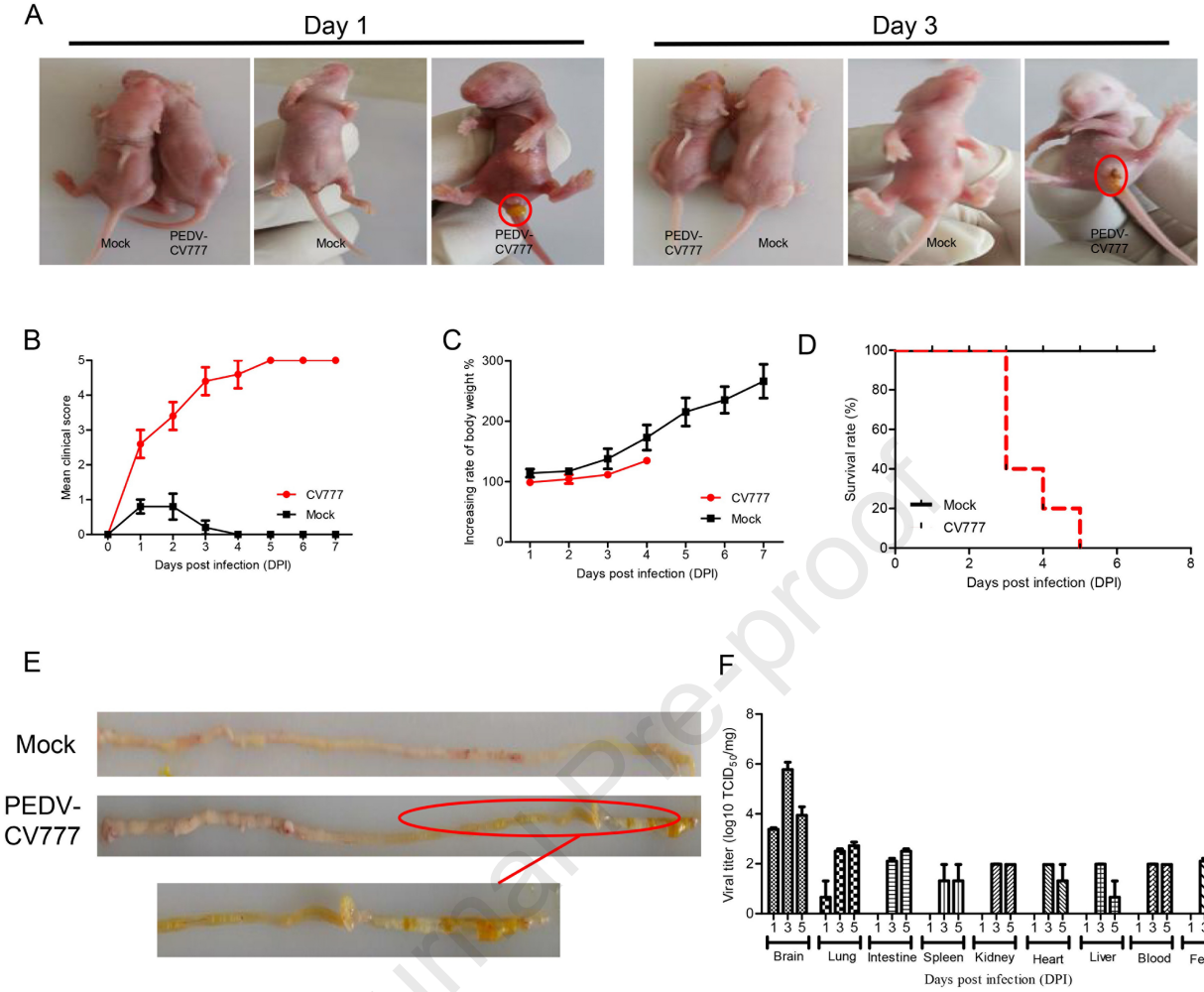


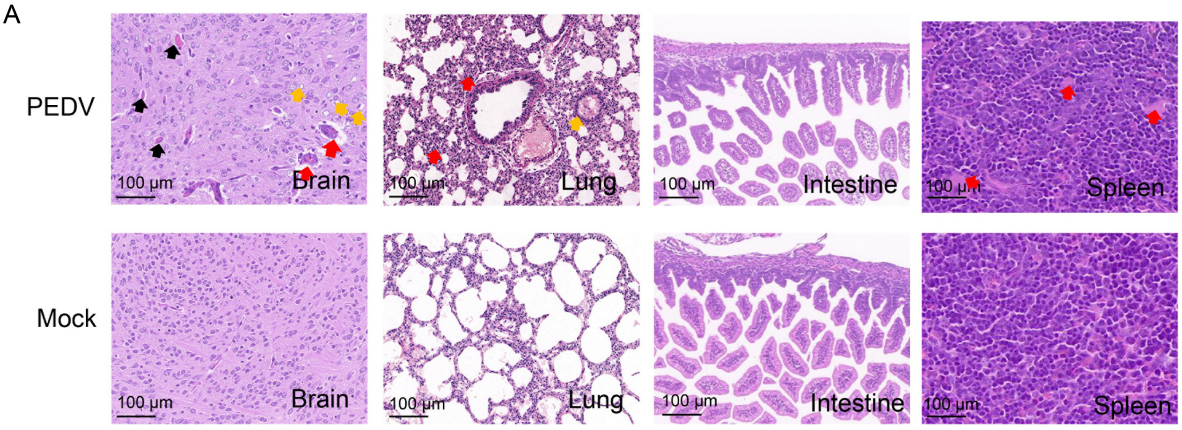


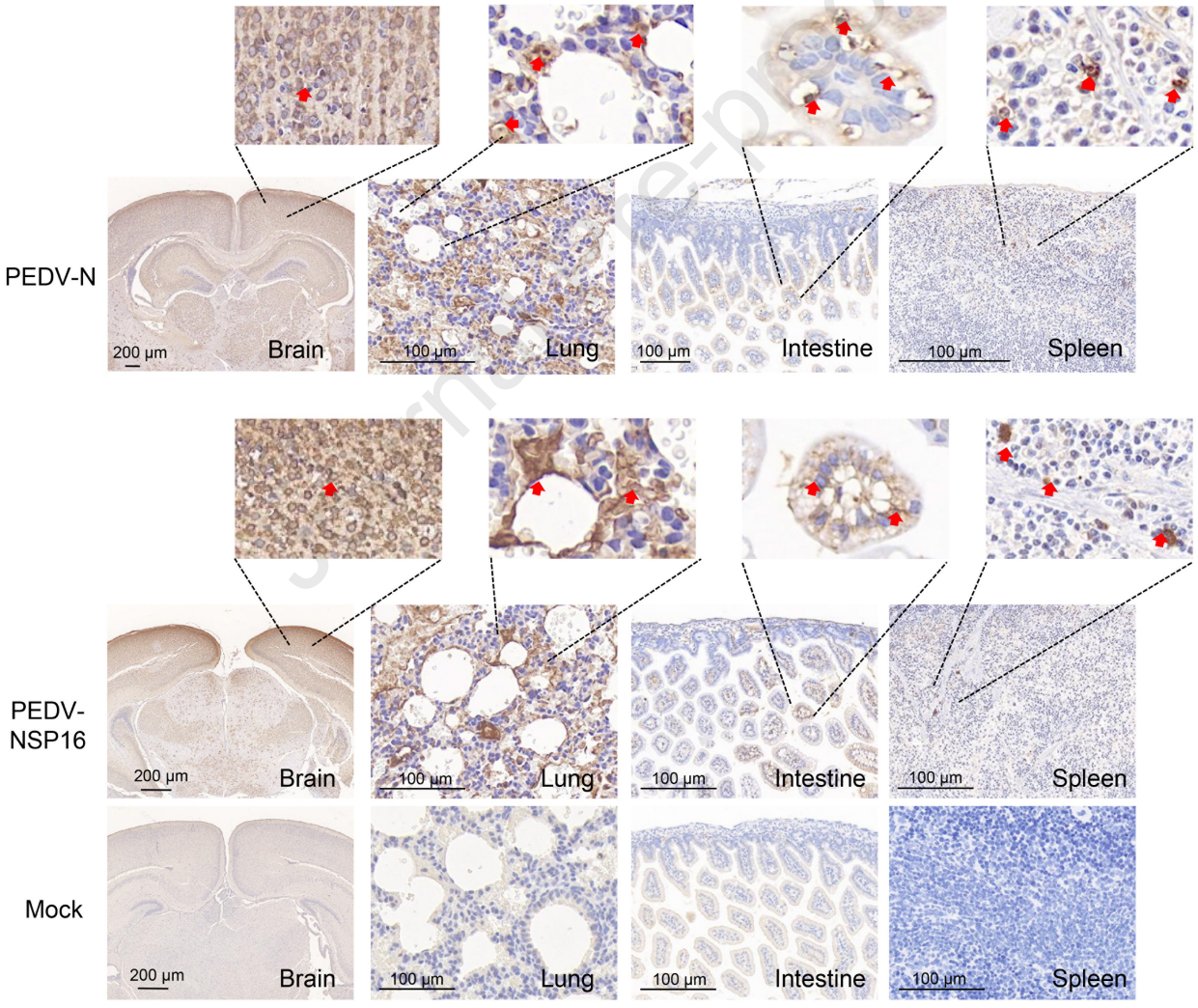


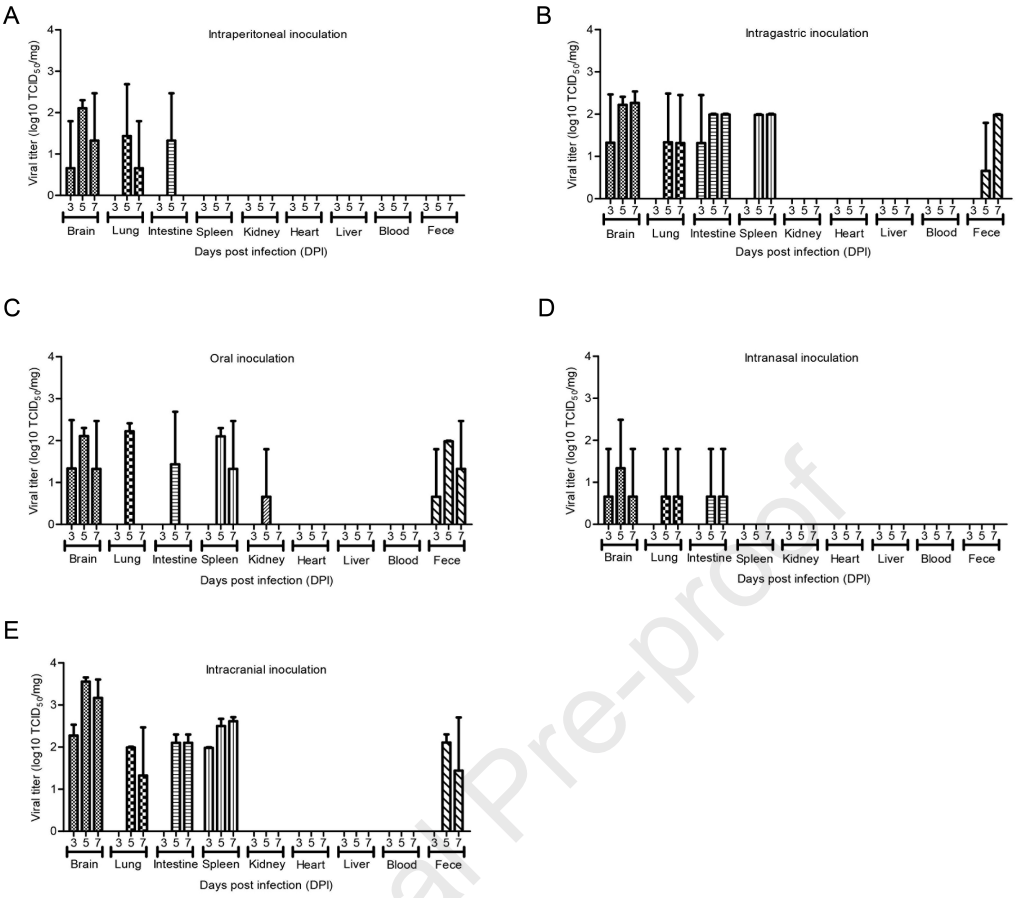












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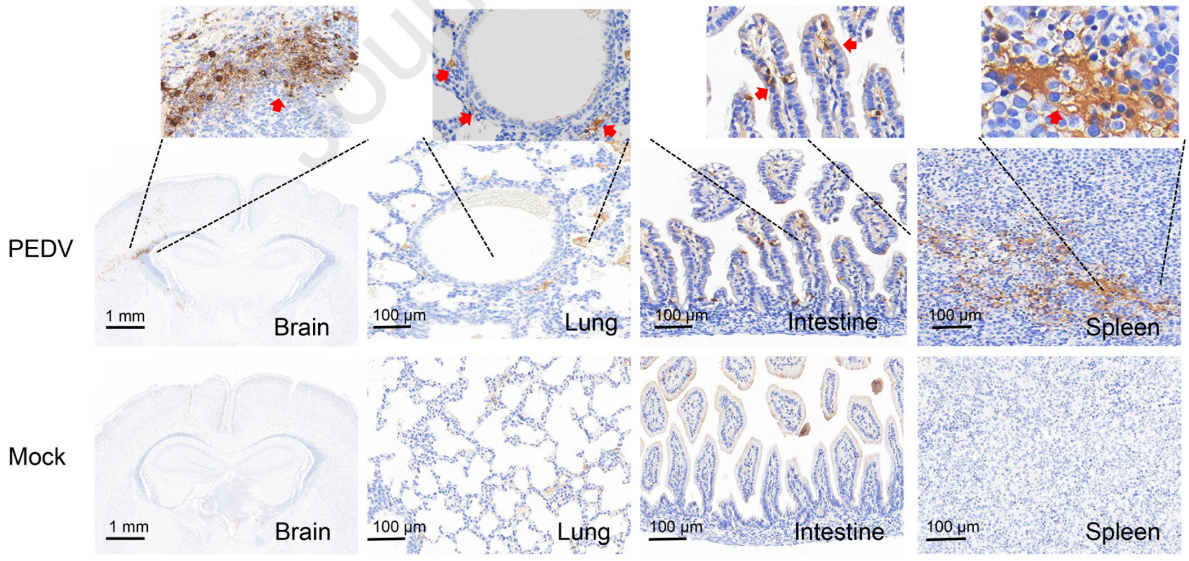

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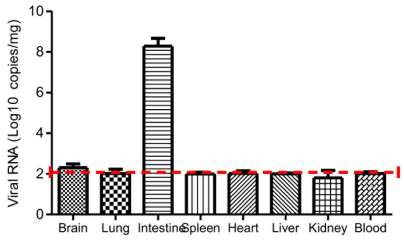

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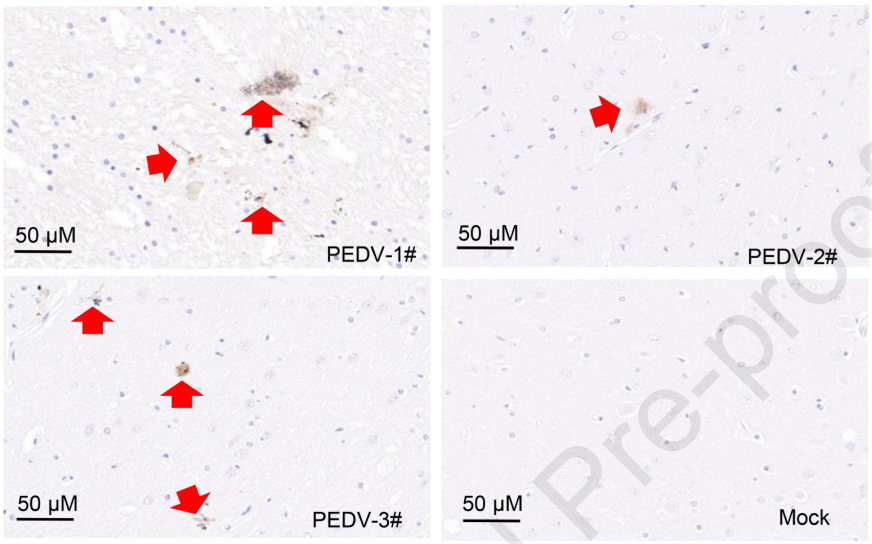

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