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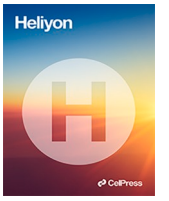

Research article

Infectivity and pathogenesis characterization of getah virus (GETV) strain via different inoculation routes in mice

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ABSTRACT

In recent years, the epidemiological profile of Getah virus (GETV) has become increasingly serious, posing a huge threat to animal and public health in China. GETV can cause multi-species infection, including horses, pigs, rats, cattle, kangaroos, reptiles and birds. However, there were few reports on the efficiency of the virus entering the host via routes of different systems. In the present study, a GETV strain (SC201807) was obtained from a piglet's blood in 2018 in Sichuan, China. First, we established a quantitative real-time polymerase chain reaction (qRT-PCR) SYBR assay specific to GETV. Then, we evaluated the infection efficiency of different routes using mouse animal model. 108 male mice were randomly divided into four groups as follows: intramuscular, intraoral and intranasal infection routes, and negative control. All mice in the experimental group were inoculated with $4 \times 10^{2.85}$ TCID50 GETV virus. Tissue tropism experiments show that GETV has a wide range of tissue distribution, and intramuscular infection is the first to infect all tissues of the body, and suggest that oral infection may be a new GETV transmission route. Histopathological examination results showed that intramuscular injection of GETV mainly caused different degrees of pathological damage to the tissues, and could rapidly induce a large amount of inflammatory regulatory factors such as IL-6 and TNF-α. Our data may help us to evaluate the risk of transmission of Porcine Getah virus and provide an experimental basis for the prevention and control of Porcine Getah virus.

1. Introduction

Alphavirus is a mosquito-borne RNA virus that usually causes acute illness, including mild fever, rash, joint pain, myalgia, and more severe encephalitis [1]. Getah virus (GETV) is a re-emerging alphavirus belonging to the alphavirus genus of the Togaviridae family that has the potential to infect humans [2,3]. GETV was first isolated from Culex mosquitoes collected in Malaysia in 1955, and has since been found in mosquitoes from Asian countries around the Pacific [4]. China's first Getah virus M1 strain was isolated from Culex

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mosquitoes in Hainan Province in 1964[5]. Before 2006, GETV was found in only six provinces in China [6], and the provinces affected by GETV in 2018 (municipalities, autonomous regions) increased sharply to 16[7, 8, 9, 10]. We must pay attention to the influence of GETV on China.

It has long been believed that GETV is only pathogenic in pigs and horses [11,12], but blue fox infection with GETV was found in Shandong in 2017 [9], cattle infection was reported in Northeast China in 2018 [8], and human serum samples GETV neutralizing antibodies can be detected in Ref. [13]. With the continuous expansion of its epidemic range and host range, it has brought serious losses to my country's pig industry and constituted a huge threat to public health. For these reasons, the potential spread of the virus and the efficient route of virus entry into the host are considered issues of concern. Reports on GETV have mainly focused on virus isolation and identification. To our knowledge, there have been no reports of mice being infected with GETV through different routes of inoculation. In this study, the pathogenicity of GETV (strain SC201807) obtained in 2018 from a piglet from a pig farm in Sichuan, China was investigated by different infection routes. Our results showed that intramuscular, intranasal, and intraoral routes of infection were effective, but muscle infection was the most effective, and suggested that oral infection may also be a new route of GETV transmission. This study also used the lesion degree and distribution of different organs after intramuscular injection to infect mice, as well as the immune response after infection, in order to further clarify the pathogenesis of virus-infected mice.

2. Material and methods

2.1. Establishment of a rapid GETV qRT-PCR SYBR detection method

2.1.1. Virus and animals

GETV SC201807 strain, which was isolated and identified and preserved by the Animal Biotechnology Center of Sichuan Agricultural University (The obtained GETV SC201807 strain was plated to BHK-21 cells in T25 tissue flask. When the cell density reached 80 %, 100 μL of 100 TCID50 dose of GETV was inoculated into BHK-21 cells, and the virus culture medium was collected 24 h later). The others viruses, including Porcine Parvovirus (PPV), Porcine Epidemic Diarrhea Virus (PEDV), Pseudorabies Virus (PRV), Porcine Circovirus Type 3 (PCV3), and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), as well as *Escherichia coli* DH5α, were provided by the Animal Biotechnology Center of Sichuan Agricultural University. SPF grade Kunming mice were purchased from Chengdu Dashuo Experimental Animal Center.

2.1.2. Design and synthesis of detection primers

Referring to the Cap gene sequence of SC201807 strain, Sangon Bioengineering (Shanghai) Co., Ltd. Used Primer Premiers 5.0 software to design a pair of specific primers (Primer sequence: F: CTTGACGGTAAGGTCACGGG; R: GTAAGCTTCGCTAGGTCGGG).

2.1.3. Preparation of GETV qRT-PCR SYBR standards

Viral RNA was extracted from the SC201807 using Trizol reagent according to the manufacturer's instructions. Reverse transcription was performed according to the instructions of the Prime Script RTKit reverse transcription kit. Use the reverse-transcribed cDNA product as a template. The program was as follows: 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 7 min. The PCR products were visualized by 1 % agarose gel electrophoresis. Then recover the GETV Cap amplified fragment according to the instructions of the SanPrep Column DNA Gel Recovery Kit (NO. SK8131) of Sangon Bioengineering (Shanghai) Co., Ltd., and store the obtained DNA solution at −20 °C for future use. The connection and transformation of target genes with plasmids should be carried out according to the instructions of the TAKARA company's pMD19-T manual.

Identification of Recombinant Plasmids: Take 50 μL of prepared bacterial solution and spread it onto an LB agar plate containing Amp+ (50 μg/mL). Then, place the plate in a 37 °C constant temperature incubator and incubate for 16–24 h until a single colony grows.

Identification of recombinant plasmids: Pick a single colony and culture it overnight in 5 mL of LB liquid medium containing Amp + resistance. Verify the obtained bacterial liquid by PCR, extract the plasmid from the identified positive bacterial liquid, and send the extracted recombinant plasmid to Sangon Technology Co., Ltd. for sequence determination and validation.

2.1.4. Establishment of GETV qRT-PCR SYBR reaction conditions

Optimization of Annealing Temperature: according to the recommended 20 μL system of Takara SYBR® Premix ExTaq™ II (Tli RNaseH Plus) kit, a plasmid standard pMD-GETV-Cap with a concentration of 1.696×10^7 copies/μL was selected for annealing temperature optimization experiment. Based on the recommended dissociation temperature during primer synthesis and design, the optimization temperature range for GETV annealing was set as 66 °C, 65.6 °C, 64.8 °C, 63.7 °C, 62.4 °C, 60.9 °C, 59.4 °C, 57.9 °C, 56.6 °C, 55.6 °C, and 55 °C to establish the optimal annealing temperature. The reaction program was set as follows: 95 °C/3 min; 95 °C/10 s; 55–66/15 s, for 40 cycles.

Establishment of Standard Curve: the recombinant plasmid standard pMD-GETV-Cap was selected and diluted into 8 gradients ranging from 1.696×10^{10} copies/μL to 1.696×10^3 copies/μL. qRT-PCR reaction was performed using an optimized method and reaction conditions. Each concentration gradient was tested in triplicate with a negative control. The amplification results from the three replicates at each gradient were averaged and used for linear analysis.

Specificity test: the specificity of the fluorescence quantitative detection method was verified by detecting GETV and other five related swine disease viruses (PEDV, PCV3, PPV, PRV, PRRSV).

Sensitivity test: the serial dilution of recombinant plasmid standard (1–11: 1.696×10^{10} copies/μL-1.696 copies/μL) pMD-GETV-Cap was used for qRT-PCR reaction, the number of qRT-PCR reaction cycles was 40, and the negative control was set. Sensitivity analysis was performed on the final results to verify the sensitivity of the method.

repeatability test: four different concentrations of GETV recombinant plasmid standards (1.696×10^{8}copies/μL, 1.696×10^{7}copies/μL, 1.696×10^{6}copies/μL, 1.696×10^{5}copies/μL) were selected for qRT-PCR, and each concentration in each assay was set in triplicate. The mean and coefficient of variation of Ct values obtained were analyzed and the repeatability was analyzed.

2.2. Comparative assessment of GETV (SC201807) pathogenicity in mice using different inoculation routes

2.2.1. SC201807 strains

The GETV SC201807 strain was used for experimental infection of Kunming mice. In a previous study, we detected and isolated a strain from the blood of GETV-positive piglets, and named it SC201807(7).

2.2.2. Experimental design

First 108 3-week-old male Kunming mice whose oral and anal swabs were negative for GETV were randomly divided into four groups: intramuscular (group A), intraoral (group B), and intranasal (group C) infection routes, and negative control (group D). Physical isolation was implemented between the experimental groups, and strict biosafety measures were taken to prevent cross-infection between groups. Food and water were freely available to the mice throughout the experimental period without the application of medicinal additives or vaccines. A and C groups were inoculated with $4 \times 10^{2.85}$ TCID50 each. And add $4 \times 10^{2.85}$ TCID50 dose of GETV virus solution to the water dispenser in group B, remove the contaminated water dispenser after 24 h. The experimental results are calculated from 24 h after the start of the experiment. Group D was the blank group without any treatment. After infection, 3 mice from groups A, C, and D, respectively, were selected at 4 h, 8 h, 12 h, 24 h, 48 h, 72 h, 7 d, 14 d, and 21 d, as well as 3 mice from group B at 24 h, 48 h, 72 h, 7 d, 14 d, and 21 d after infection. Blood was collected and serum was separated and stored for future use. The mice were then slaughtered humanely, and a portion of their organs (liver, spleen, lungs, kidneys, mesenteric lymph nodes, stomach, small intestine, large intestine, cerebrum, cerebellum, and testes) were collected in 4 % buffered formalin for histopathological examination. Another portion of the organs was stored in liquid nitrogen for GETV qRT-PCR detection.

Then in order to study the immune response of mice infected with SC201807 strain, 94 3-week-old male Kunming mice were randomly divided into infection group and control group. The infection group was injected intramuscularly with 100 μL of GETV virus solution at a dose of $4 \times 10^{2.85}$, and the control group was injected intramuscularly with the same volume of PBS. At 0 h, 2 h, 4 h, 8 h, 12 h, 24 h, 36 h, 48 h, 72 h, 7 d, 14 d, 21 d, 28 d, 35 d, and 42 d post-infection, 3 randomly selected animals from each group were used to collect blood samples for determination of viral load and cytokine levels, as well as neutralization assays.

2.2.3. Tissue distribution, virus shedding and viremia law

All above tissues, serum, and oropharyngeal and cloacal swabs were collected and stored at −80 °C until RNA extraction. RNA was extracted, and reverse transcription and qRT-PCR were performed as above to test the virus shedding. In addition, cDNA of the isolate in this study and reagent-grade water were used as positive and negative control, respectively.

2.2.4. Histopathological examination

Tissues from each group were collected and fixed with 4 % Paraformaldehyde. The sections were stained with hematoxylin and eosin (HE), and all HE stained sections were examined for the presence of microscopic lesions.

2.2.5. Neutralization test

After inactivating the collected serum samples at 56 °C for 30 min, they were diluted 2, 4, 8, 16, 32, 64, 128, and 256 times. The diluted serum was then mixed 1:1 with 200 TCID50 virus liquid, and the mixture was incubated at 37 °C for 1 h. The mixture was then inoculated onto a 96-well plate (BHK-21) with four replicates per dilution. Normal cells, GETV-infected cells, and high-concentration serum-inoculated cells were used as controls. After 48 h of incubation, the cytopathic effect (CPE) was observed and the endpoint of 50 % serum neutralization was calculated using the Reed-Muench method.

2.2.6. Cell factor measurement

The collected blood was left at 4 °C overnight, the blood clots were fully collected, the heart was 3000 r/min, the serum was sucked, and the blood was stored at −80 °C after dispensation. Please refer to the mouse source ELISA reagent sheet for reference, and study the horizontal changes in mouse IL-2, IL-6, TNF-α and IFN-γ expression.

2.2.7. Statistical analysis

All experimental data were statistically analyzed using SPSS, and the statistical differences between the two groups were analyzed using the independent sample *t*-test (Student's two-tailed unpaired *t*-test). The results were expressed as mean ± SD, and the graphics were completed with GraphPad Prism 8.1.

3. Results

3.1. pMD-GETV-Cap plasmid standard RT-PCR identification

The results showed that the concentration of the constructed pMD-GETV-Cap plasmid was measured to be 587.64 ng/μL, and the plasmid standard was used as a template, and Cap-F/Cap-R primers were used for RT-PCR amplification identification, and the target of about 316 bp was obtained (Fig. 1A). The fragments were compared by BLAST on NCBI, which showed that the gene homology of the sequence was 100 %, indicating that the constructed plasmid standard conformed to it, which was convenient for the application of subsequent experiments.

3.2. Optimization of qRT-PCR SYBR reaction conditions

3.2.1. Optimization of annealing temperature

Through the annealing temperature optimization experiment, the optimal temperature is 57.9 °C (Fig. 1B). According to the optimized annealing temperature, the reaction program of qPCR was determined as follows: 95 °C, 3 min; 95 °C, 10 s; 57.9 °C, 15 s, 40 cycles.

3.2.2. Establishment of standard curve

The figures below is the standard curve (Fig. 1C) of the qRT-PCR standard template. In the concentration range of 1.696×10^{10} copies/μL -1.696×10^{3} copies/μL, the linear equation of the obtained copy number (x) and Ct value (y) is $y = -2.8611x+37.14$, the coefficient of determination $R^2 = 1.00$, showing have a good linear relationship.

3.2.3. Specificity test

The GETV fluorescent quantitative detection method was utilized for detecting GETV along with five other swine disease viruses (Fig. 1D). Results revealed that only GETV exhibited amplification of the curve, while the other five related swine disease viruses (PEDV, PCV3, PPV, PRRSV, PRV) did not demonstrate curve amplification. These findings underscore the excellent specificity of the established real-time RT-PCR detection method for GETV.

3.2.4. Sensitivity test

Performing a qRT-PCR reaction on the serially diluted recombinant plasmid standard (1–10:1.696×10^{10}copies/μL-1.696copies) pMD-GETV-Cap, the established GETV fluorescent quantitative RT-PCR detection method can detect a minimum standard sample concentration of 16.96 copies/μL (Fig. 1E).

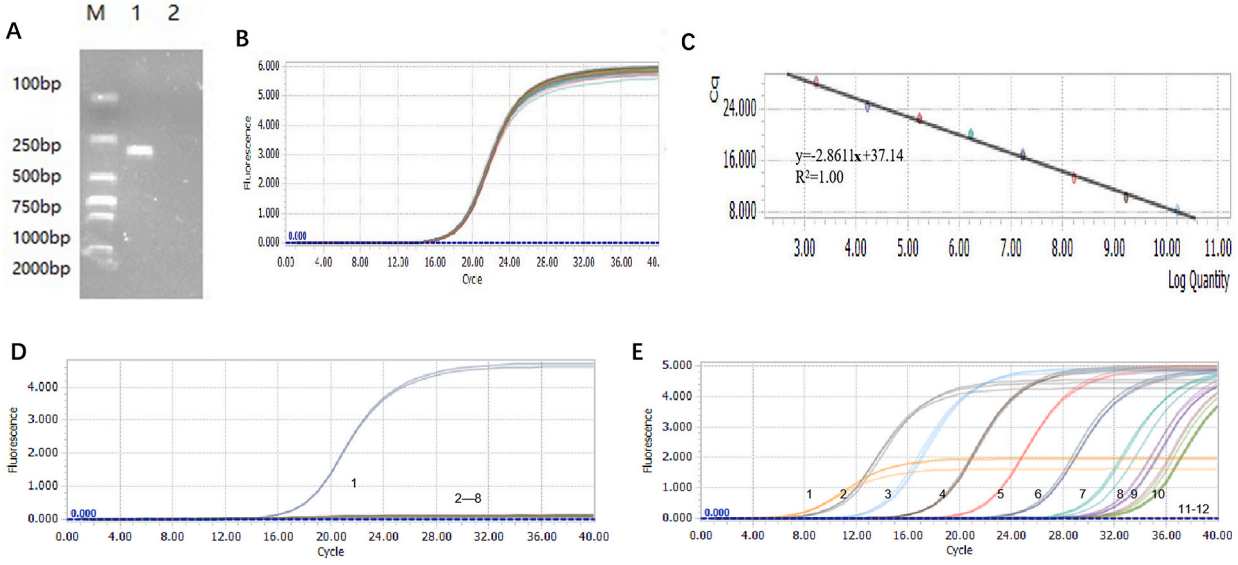

Fig. 1. Optimization of the SYBR reaction conditions for GETV qRT-PCR. A: RT-PCR results of GETV-Cap plasmid standard (M: DL2000; 1: plasmid standard RT-PCR products; 2: negative control); B: Optimal temperature for the GETV qRT-PCR SYBR (Amplified graph); C: qRT-PCR SYBR draws a standard curve based on the Ct value of the amplification curve and the initial copy number of the standard plasmid; D: GETV qRT-PCR SYBR fluorescence quantitative PCR specificity test (1: GETV; 2–6 in turn are: PEDV, PCV3, PPV, PRV, PRRSV; 7, 8: Negative control). E: GETV qRT-PCR SYBR fluorescence quantitative PCR sensitivity test (1–11: Plasmid standards are: 1.696×10^{10} copies/μL - 1.696 copies/μL; 12: negative control).

3.2.5. Test of repeatability

The coefficient of variation (CV%) values of four different concentrations of GETV (1.696×10^8 copies/μL, 1.696×10^7copies/μL, 1.696×10^6 copies/μL, 1.696×10^5copies/μL) recombinant plasmid standards were less than 2.5 %. The results showed that the established real-time RT-PCR detection method of GETV had good repeatability and stability (Table 1).

3.3. Comparative assessment of GETV (SC201807 strain) pathogenicity in mice using different inoculation routes

3.3.1. Tissue tropism and the duration of viral sepsis of the SC201807 in different routes of infection

In this study, we found that the virus can effectively replicate in different tissues, no matter which route of infection. Within 24 h of infection, the proliferation rate of viral load in the brain was the highest in the intramuscular injection mode; the highest proliferation rate in the stomach was in the intraoral infection mode; the highest proliferation rate in the lung tissue was in the intranasal infection mode. The viral load in the blood of mice infected by intramuscular injection reaches a peak at 24 h, and virus presence can still be detected 21 days after infection, indicating a viremia duration of more than 21 days following intramuscular infection(Figss. 2–4).

3.3.2. Virus shedding

The results showed that mice shed virus from 24 h to 21d. The earliest start time observed was in group A (intramuscular infection) in 24 h. The highest shedding rates were observed in group B (intraoral infection) from the throat at 7d and cloaca at 24 h.The highest shedding rates were observed in group C (intranasal infection) from the throat and cloaca at 72 h. Viral shedding ceased at 21d in all groups (Fig. 5).

3.4. Further research on the effect of intramuscular injection of GETV (SC201807 strain) on mice

3.4.1. Histopathological examination

The results showed that there was no clear boundary between the cortex and medulla of the mesenteric lymph nodes in the injected group, with an increased number of lymphocytes and difficulty in distinguishing lymphoid nodules in the cortex; a small number of lymphocytes in the medulla showed degeneration and necrosis, with cell disintegration and blurred structure, and occasional macrophages were observed (Fig. 6A). Local necrosis was observed in both the cerebrum and cerebellum of the brain tissue, with obvious cytoplasmic vacuolization and some nuclear shrinkage in the necrotic area (Fig. 6B–F). A few liver cells showed focal necrosis, with blurred cell structure, nuclear shrinkage, and fragmentation (Fig. 6C). The seminiferous epithelium of the testis, including supporting cells, spermatogonia, spermatocytes, and sperm cells, was loosely arranged, and few sperm were observed in the lumen of the tubules (Fig. 6D). The white pulp splenic follicles were expanded, and there was a significant increase in lymphocytes in the marginal zone (Fig. 6E). No significant pathological changes were observed in the mesenteric lymph nodes, cerebrum, cerebellum, liver, testis, spleen, and other tissues in the control group (Fig. 6G, H, I, J, K, L).

3.4.2. Anti-GETV antibody titer detection

The antibody titers against the SC201807 strain are summarized in Fig. 7. There was an increase in antibody levels throughout the experimental period in challenged group as compared to the negative control mice.

3.4.3. Cellular factor measurement result

After intramuscular infection for 2 h, the concentration of IL-6 and TNF-α in the experimental group were higher than those in the PBS group, while the concentration of IL-2 was lower than those in the PBS group. It can be seen that GETV infection promotes the production of IL-6 and TNF-α in mice and inhibits the expression of IL-2. Before intramuscular infection for 6 h, the concentration of IFN-γ was lower than that of the control group. After intramuscular infection for 6 h, the concentration of IFN-γ first increased and then decreased, and was higher than that of the control group (Fig. 8).

4. Discussion

GETV is an arbovirus, and Li Yuanyuan et al. [13] conducted a serological investigation of GETV in domestic animals in Yunnan Province and found that its GETV antibodies were detected in serum samples from chickens, ducks, dairy cows, pigs and beef cattle, suggesting that there may be a large number of GETV host animals in the area, and the mechanism of vector transmission causing infection among multiple animals is also unclear. The known mode of transmission is mosquito bite, but the existence of other modes of

Table 1
qRT-PCR SYBR repeatability test.

Sample concentration (copies/μL)	intra-assay variation		inter-assay variation	
	X±SD	CV(%)	X±SD	CV(%)
1.696×10^8	13.19 ± 0.04	0.3	13.10 ± 0.13	0.97
1.696×10^7	16.75 ± 0.04	0.24	16.79 ± 0.06	0.34
1.696×10^6	19.92 ± 0.01	0.05	20.19 ± 0.37	1.86
1.696×10^5	22.46 ± 0.09	0.4	22.83 ± 0.52	2.26

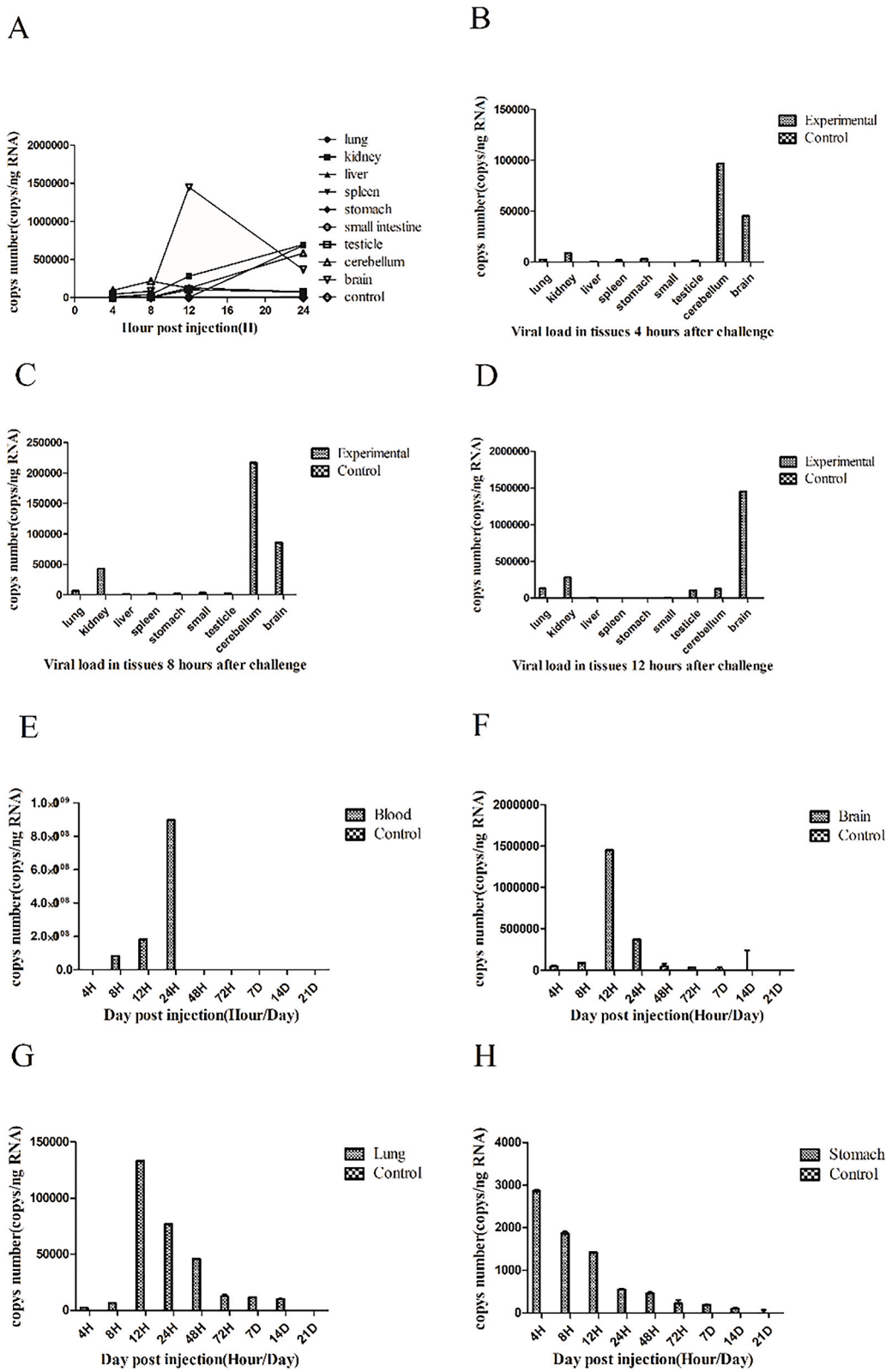

Fig. 2. Results of viral load in tissue and blood after intramuscular injection. A: Viral load in all tissues before 24 h after treatment; B: Viral load in tissues 4 h after treatment; C: Viral load in tissue 8 h after treatment; D: viral load in tissues 12 h after treatment; E: Viral load in blood after treatment; F: viral load in the brain after treatment; G: Viral load in the lungs after treatment; H: Viral load in the stomach after treatment.

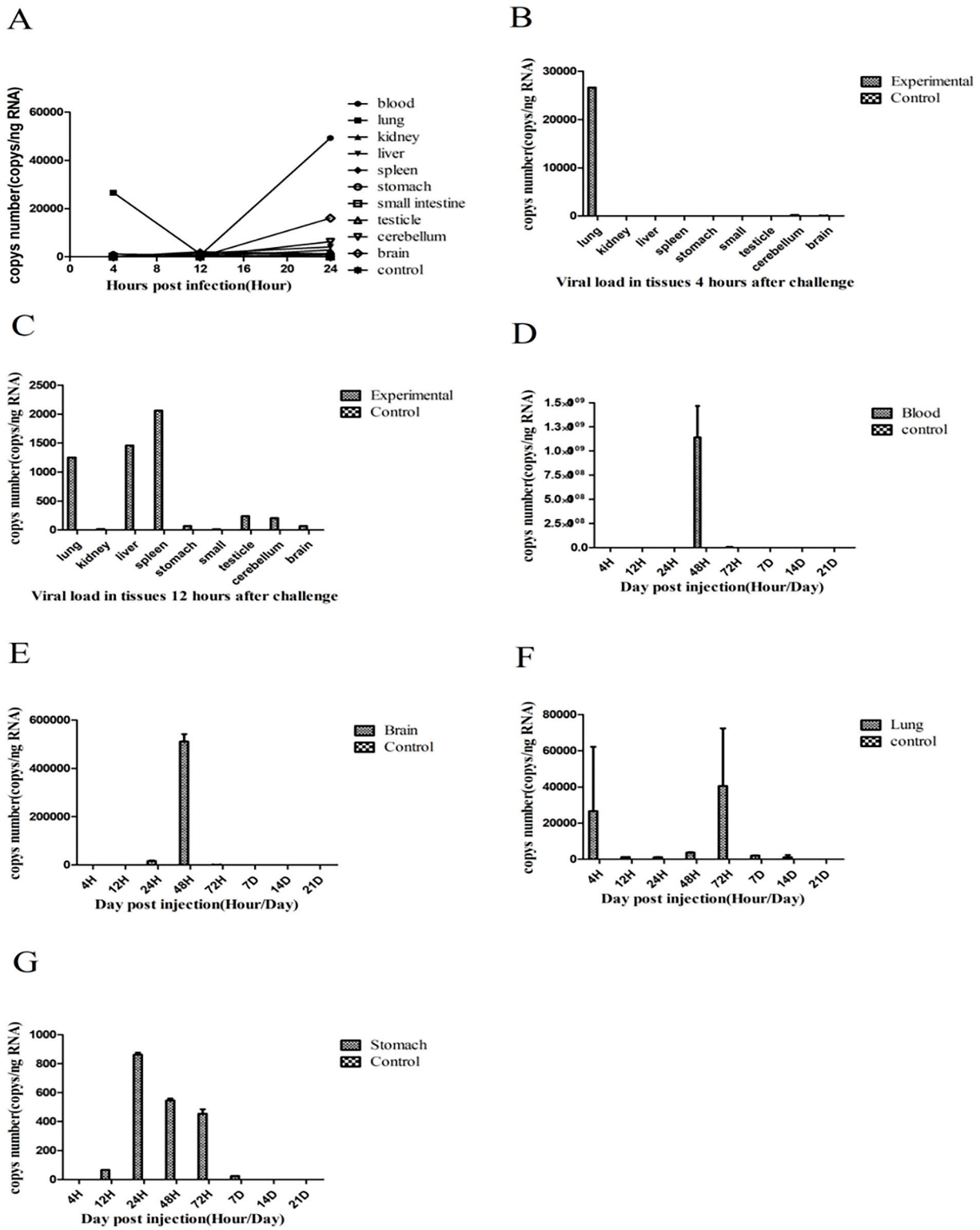

Fig. 3. Results of viral load in tissues and blood after intranasal challenge. A: Viral load in all tissues before 24 after challenge; B: Viral load in tissues 4 h after challenge; C: Viral load in tissues 12 h after challenge; D: Viral load in blood after challenge; E: viral load in the brain after challenge; F: Viral load in the lungs after challenge; G: Viral load in the stomach after challenge.

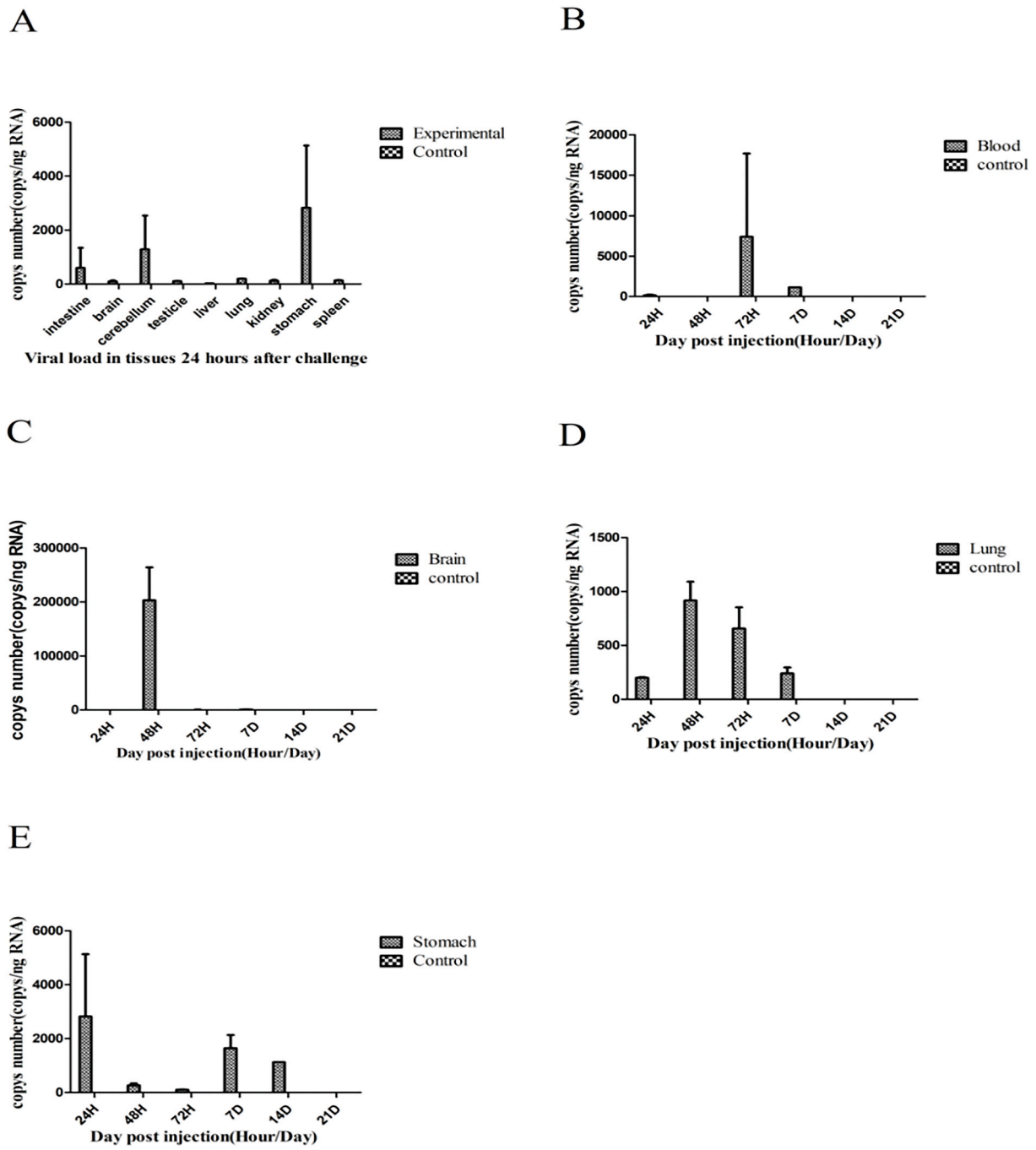

Fig. 4. Results of viral load in tissues and blood after intraoral challenge. A: Viral load in tissues 24 h after challenge; B: Viral load in blood after challenge; C: viral load in the brain after challenge; D: Viral load in the lungs after challenge; E: Virus load in the stomach after challenge.

transmission and whether hosts are cleared after infection with the virus have not been reported. The present study provides detailed findings highlighting the role of different infection pathways in mice experimentally infected with GETV over the course of time in pathogenesis. Intramuscular inoculation is a common method for evaluating viral infection. Intraoral infection mimic was used to simulate contaminated food and water. Intranasal infection was often used by labs as a substitute for aerosol infection of the virus. It is worth stating that molecular detection of the virus remains one of the most important steps in diagnosis of the virus [14]. Therefore, we have successfully established a qRT-PCR SYBR detection method for Porcine Getah virus after condition optimization.

The ORF at the 3′ end of GETV encodes five structural proteins, including the C protein, E3 protein, E2 protein, 6 K protein, and E1 protein [15]. The Cap protein is the viral shell protein, which has high structural stability and is crucial for the formation and survival

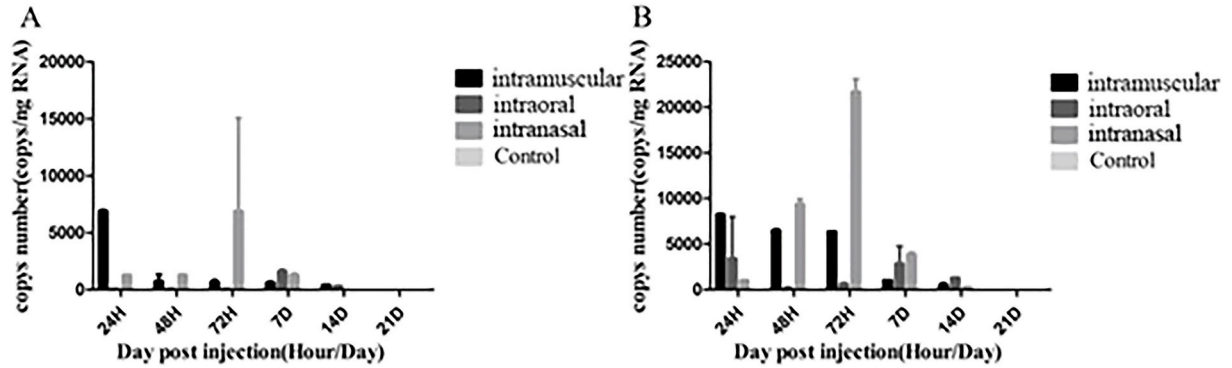

Fig. 5. Virus Shedding of throat and cloaca in Different Routes of Infection. A: Viral load in the oropharyngeal swabs; B: Viral load in the cloacal swabs.

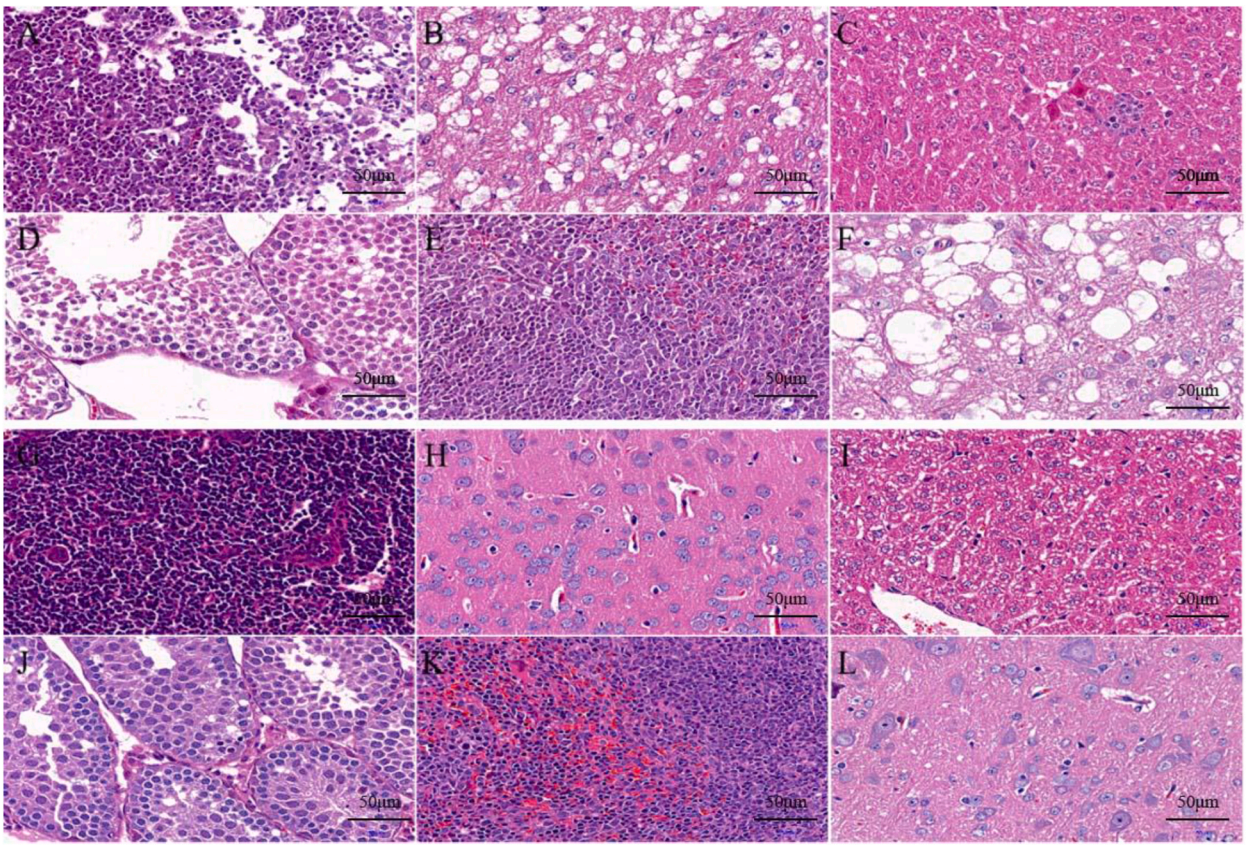

Fig. 6. Histopathological changes of mice (HE × 400). A–F: challenge group: mesenteric lymph nodes, brain, liver, testis, spleen, cerebellum; G–L: control group: mesenteric lymph node, brain, liver, testis, spleen, cerebellum.

of virus particles [16]. In this study, a pair of specific primers was designed for the conserved region of the GETV Cap gene, and a qRT-PCR SYBR detection method for porcine GETV was successfully established through optimized conditions to accurately, sensitively, and efficiently detect large batches of samples for the purposes of pig introduction, early diagnosis, virus monitoring, and in-depth virus research. It was verified that the method had good specificity and no cross-reaction with other viruses in our laboratory, such as PEDV, PCV3, PPV, PRV and PRRSV. The sensitivity experiment showed that the lowest detectable concentration of plasmid standard was 16.96 copies/μL. Wang et al. [17] established a conventional RT-PCR method for GETV, with a minimum detectable titer of $10^{2.25}$ TCID50/ml; Guan et al. [18] established a dual RT-PCR method for porcine atypical pestivirus and GETV, with the lowest detectable nucleic acid detection amounts for both viruses being 187 copies/μL and 126 copies/μL, respectively; this method is 10–100 times more sensitive than conventional PCR, indicating that qPCR has higher sensitivity than conventional PCR. Sam et al. [14]

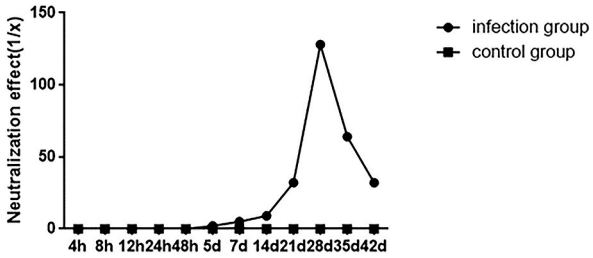

Fig. 7. Neutralizing antibody test results of GETV.

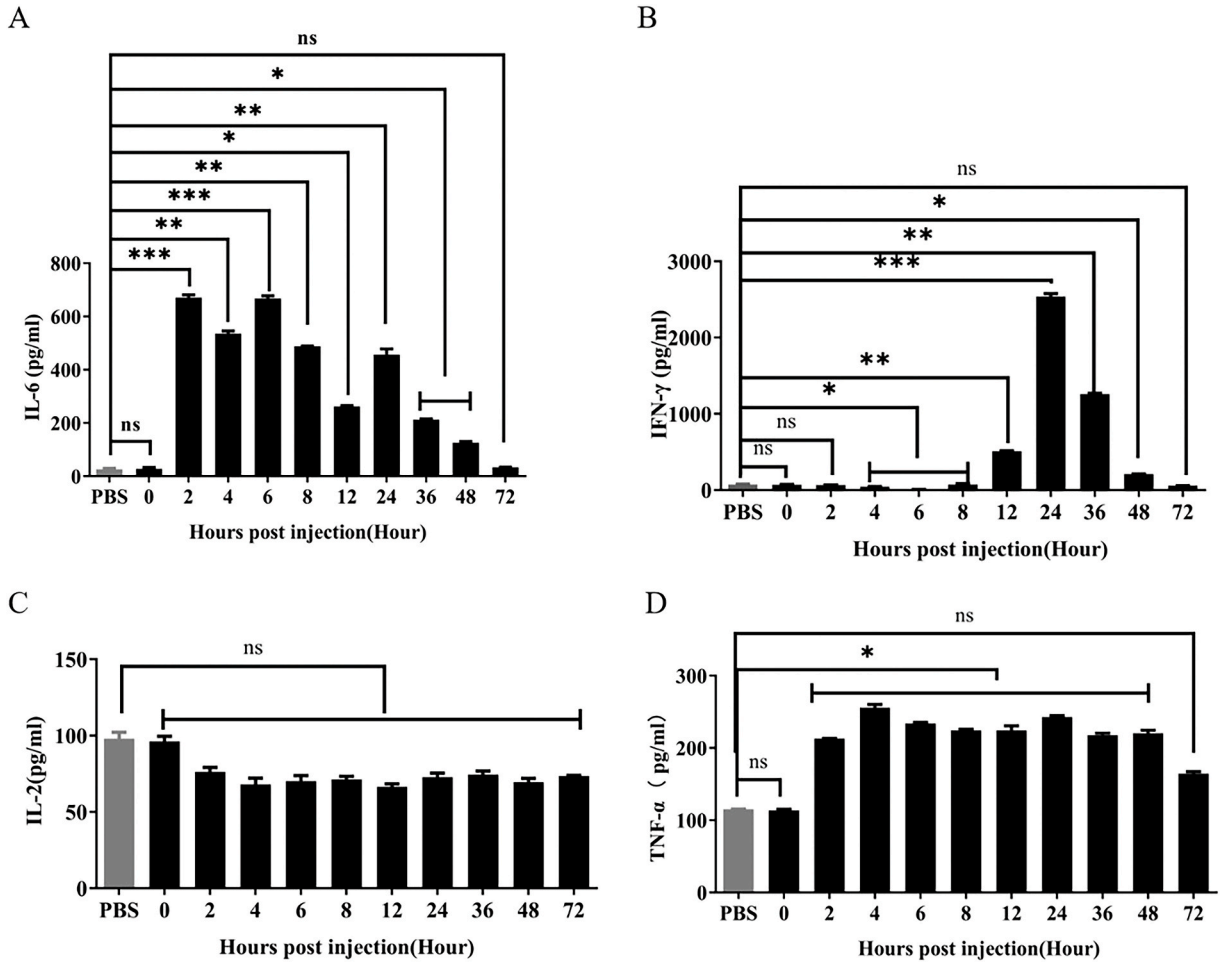

Fig. 8. Cytokine ELISA results of each group of animals. Note: All results are shown as mean ± SD; PBS, ELISA results of negative control group; 0, ELISA results before the mouse challenge; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, no significant difference.

established a GETV TaqMan MGB probe method, with the lowest detectable nucleic acid concentration being 63.25 copies/μL; Zhu et al. [19] established a TaqMan probe fluorescent quantitative RT-PCR detection method, with the lowest detectable nucleic acid concentration being 20.3 copies/μL; the sensitivity of the method established in this study was found to be stronger. The repeatability of the GETV qRT-PCR SYBR detection method established in this study was good and relatively stable, with intra-group and inter-group variation coefficients (CV%) of less than 2.5 %. This method has good application value in screening for GETV in field samples and helps to understand the infection status of GETV and monitor the disease. It can provide technical support for the detection of GETV viral load and the exploration of tissue tropism and transmission characteristics in the future.

In our study, we used the above-designed qRT-PCR detection method to measure the viral load in different tissues of mice infected with GETV through intramuscular injection, intraoral infection, and intranasal infection. The results showed that all three infection methods could detect the presence of GETV in all tissues within 24 h of infection, but the order of tissue invasion by different infection

methods was different. Li [20] showed that after intramuscular injection of GETV in mice, the virus appeared in the testicular and caused testicular histopathological changes and decreased semen quality and testosterone levels, which is consistent with our findings. Yang [21] also found that GETV was detected in multiple organs of getv-infected piglets, indicating that GETV can infect different species with multiple tissue tropisms. The results of this study showed that GETV could be detected in all tissues of mice infected by three modes of infection, but the sequence of virus appearance in each tissue was: intramuscular infection group, intranasal infection group and oral infection group.

In order to explore the distribution of the virus in tissues and blood over a period of time, the cerebellum, lungs and stomach of the mice were observed for up to 21 days. In the viral load measurements lasting 21d, it was found that the three different modes of infection had correspondingly different effects on the viral load in tissues, the duration of viremia, and the duration of virus persistence in tissues. The viral load in the blood and most tissues of the mice peaked at 24 h during the intramuscular infection. After 48 h intranasal infection in mice, the viral load in the blood reached its peak, with a duration of approximately 21 days. Following oral infection, the peak viral load in the blood was observed after 72 h, with a duration of approximately 14 days. Similarly, horses and pigs are also able to produce high enough viremic titers to infect biting mosquitoes and sustain GETV transmission cycles [11,22]. This study can provide some ideas for the prevention and control of GETV.

Next, we compared the viral load of GETV in oral and fecal swabs after infecting using three different routes of infection, and found that all three routes resulted in viral shedding in the oral and fecal samples. However, the patterns of viral shedding in the oral and fecal samples differed depending on the route of infection, and the fecal shedding was consistently higher than oral shedding for all three routes of infection during the 21-day monitoring period, the viral load of GETV in oral and fecal swabs is changing over time and the highest oral shedding (total) is in the intranasal injection group. In the intramuscular injection group, the viral load in oral and fecal samples peaked 24 h after infection, and the shedding period lasted for 21 days in both oral and fecal samples. In the intraoral infection group, the viral load in oral and fecal samples peaked 7 days after infection, but the shedding period lasted for 21 days in fecal samples and only 14 days in oral samples. In the intranasal infection group, the viral load in oral and fecal samples peaked 72 h after infection, and the shedding period lasted for 21 days in both samples. These results indicate that intramuscular injection results in early and high viral shedding, intranasal infection results in mid-term and high viral shedding, while oral infection results in late and relatively short shedding periods. The intraoral infection route resulted in the lowest viral shedding and shortest shedding period.

The above research found that the intramuscular injection route is the first to invade all tissues of the body, has the fastest proliferation rate and longest persistence time. It poses the greatest harm and has the most significant impact on the body. Additionally, it releases a large amount of virus through oral and fecal excretion in the early stages of infection, with a prolonged excretion period. Therefore, we conducted further research on intramuscularly infected mice.

Observation of mice intramuscular infected with GETV for 72 h by pathological sections revealed that it caused infiltration of inflammatory cells such as lymphocytes and macrophages in joints, brain, liver, spleen and lymph nodes, while partial cell necrosis was seen in liver, brain and joint tissues, indicating that GETV infection caused different degrees of inflammatory responses in these tissues. In other mice models of Sindbis virus (SINV) and Chikungunya virus (CHIKV) infection showed brain cell necrosis and joint damage as an immunopathological inflammatory disease [23–25], suggesting that inflammatory cells such as macrophages, lymphocytes, and multinucleated giant cells infiltration after GETV infection may be involved in the pathogenesis of its induced encephalitis and arthritis.

Intramuscular infection can trigger inflammatory reactions in tissues. These outcomes have drawn our attention. Cytokines play an important role in the process of inflammation development, and there are currently no reports on the immune regulatory role of cytokines after GETV infection. In this study, we measured the dynamics of antibody levels in the blood of mice after intramuscular infection and monitored the expression levels of cytokines in the blood of mice at different time points before and after GETV infection, to investigate the induction of the host's innate immune response after GETV infection.

Our previous results showed that the viral load of mice infected with the virus peaked at 24 h, which is consistent with the peak viral titers observed in the acute encephalitis model of mice infected with SINV between 24 and 48 h [26]. We monitored mouse antibody levels and found that anti-GETV neutralizing antibodies began to be produced as early as 48 h after infection, effectively preventing viral infection of host cells and causing a high level of neutralizing antibody production in a short period of time. The viral load gradually decreased as the antibody levels continued to rise, and the virus had been cleared from the body by day 28 when the antibody level reached 1:128, indicating that neutralizing antibodies played an important role in the late stage of inflammation (after 48 h). At day 35, the neutralizing titer was 1:64, and the level of neutralizing antibodies remained high. However, at 42 days, the neutralizing titer rapidly dropped to 1:32, possibly due to the lack of a second injection to stimulate enhanced immunity in the body.

Cytokines are important components of the body to perform immune functions and have powerful immunomodulatory and immune activating effects [27]. It has been shown that arthritis-associated metapneumovirus infection is able to induce extensive cytokine and chemokine production [28]. In this study, mice were found to respond to the acute inflammation of GETV infection by rapidly inducing the body to produce large amounts of inflammatory cytokines such as IL-6 and TNF-α, which induce natural immunity; 8 h after GETV infection, the body was stimulated to produce high levels of the immune response product IFN-γ, which activated the host's natural immunity.

The expression level of IL-2 decreased but did not differ significantly within 72 h of GETV infection. In the early stage of infection, IL-6 was expressed at a high level about 24 times higher than that of the control group, and remained significantly expressed until 48 h, and returned to normal expression level at 72 h. This indicates that IL-6 is mainly secreted by Th2 type cells in large quantities to mediate humoral immunity and regulate the body's immune response in the early stage of infection. Meanwhile, related meta virus studies found that CHIKV infection of osteoblasts with IL-6 expression may lead to bone loss, arthralgia and arthritis [29], and pathological section results also showed joint damage, speculating that the high level of IL-6 produced by GETV infection may be

responsible for the development of arthritis. Patients with severe rheumatoid arthritis show increased levels of TNF-α in both their blood and joints. Studies have indicated that similar cytokine elevations occur during influenza Alphaviruses infection as in patients with rheumatoid arthritis. As is well-known, inflammation in rheumatoid arthritis patients is associated with an autoimmune process; however, there have been no reports related to Alphaviruses [30,31]. Our results showed that TNF-α was consistently expressed in serum at levels twofold higher than controls in the early stages of infection until normal expression levels were restored at 72 h, suggesting that the body triggers an inflammatory response by regulating TNF-α expression, while rheumatoid arthritis and arthritis induced by alphavirus share a series of features that may help in the development of treatments for viral arthritis.

In early infection, IFN-γ expression in serum is first suppressed at the beginning of infection (within 8 h), and then expressed at high levels between 8 h and 24 h, with the highest expression at 24 h, 28-fold higher than in normal controls, when viremia also reaches its peak, followed by a significant decline, probably because IFN-γ plays an important antiviral role at this time, with a significant decline due to massive clearance of virus from the blood. It has been shown that the interferon (IFN) program is activated early in alphavirus infection, while alphaviruses have developed several mechanisms to antagonize this antiviral response and thus suppress the host's natural immunity, such as CHIKV induces ISG expression, but it maintains the translation of viral proteins by causing eiF2a phosphorylation via PKR, which promotes translation shutdown of the cell wide genome [32]. Therefore, IFN-γ expression was significantly suppressed at the beginning of GETV infection, and it is likely that GETV antagonizes IFN-γ through some unknown pathway, resulting in a trend of first decreasing and then increasing IFN-γ in mouse serum.

5. Conclusions

In summary, GETV infected mice by three ways: intramuscular injection, intraoral and intranasal infection, intramuscular infection was the most pathogenic for model animals, intramuscular infection infiltrated all tissues in the shortest time and caused different degrees of inflammation in the relevant tissues, we conducted a preliminary study on the induction of immune response by intramuscular infection mode, and the results showed that the early stage of GETV infection by IL-6, TNF-α respectively mediated humoral immunity, cellular immunity play a role, antiviral defense through IFN-γ, inflammation and immune response activation, and virus clearance by neutralizing antibodies in the middle and late stages of infection. At the same time, Alphaviruses infection can antagonize the expression of interferon, thus suppressing the host's natural immunity, which may be one of the reasons for persistent virus infection within 21d. Factors that may cause persistent virus infection should be considered in vaccine studies, and the number of immunizations should be increased to prolong the generation of high levels of neutralizing antibody potency. But whether swines have the same immune response remains to be determined.

Consent for publication

Our present work does not contain any individual persons' data, so it is not applicable.

Ethics statement

The animal protocols and all procedures of the experiment were performed in compliance with the laws and guidelines of Sichuan Agricultural University Animal Care and Use Committee (20230152).

Availability of data and materials

All data generated or analyzed during this study are included in the main manuscript.

CRediT authorship contribution statement

Zhijie Jian: Writing – original draft, Resources, Investigation, Formal analysis. **Chaoyuan Jiang:** Writing – original draft, Formal analysis. **Ling Zhu:** Writing – review & editing, Resources. **Fengqin Li:** Methodology. **Lishuang Deng:** Software. **Yanru Ai:** Validation, Supervision. **Siyuan Lai:** Supervision. **Zhiwen Xu:** Writing – review & editing, Resources, Investigation, Formal analysis.

Declaration of competing interest

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Appendix A. Supplementary data

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