Tk-deleted pseudorabies virus retains high pathogenicity in rats

Lirong Zhang[1*], Keyue Ruan[1*], Guoju Sang[1], Zhaoyang Xu[1],
Wu Tong[1], Hai Yu[1], Tongling Shan[1], Fei Gao[1], Liwei Li[1],
Ning Kong[1], Guangzhi Tong[1, 2], Hao Zheng[1, 2, ✉]

[1]Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200241, China
[2]Jiangsu Co-Innovation Center for the Prevention and Control of Important Animal Infectious Disease and Zoonoses,
Yangzhou University, Yangzhou 225009, China
haozheng@shvri.ac.cn

Abstract

Introduction: The pseudorabies virus (PRV) gene encoding thymidine kinase (tk) is an important virulence-associated factor. Attenuation of PRV in susceptible animals is a frequent result of tk deletion. The aim of the study was to assess the pathogenicity of tk-deleted PRV in rats. **Material and Methods:** Sprague Dawley rats were infected with the tk-deleted PRV strain SuHV-1 ΔTK:247*via* intranasal or intramuscular inoculation. PRV loads in ten tissues from dead and euthanised rats were determined using real-time PCR. **Results:**Infection with SuHV-1 ΔTK:247 could cause death in rats. The 50% lethal dose (LD_{50}) of SuHV-1 ΔTK:247 *via* intranasal inoculation was $10^{3.16}$ $TCID_{50}$ in rats. Intramuscular inoculation required a higher dose of SuHV-1 ΔTK:247 ($10^{5.0}$ $TCID_{50}$). A high SuHV-1 ΔTK:247 titre was observed in the trigeminal ganglia or spinal cord of dead rats. **Conclusion:** The results of this study show that rats are highly susceptible to PRV infection, and tk deletion did not completely diminish the pathogenicity of PRV in rats.

Keywords: pseudorabies virus, thymidine kinase, rat.

Introduction

Pseudorabies virus (PRV) belongs to the *Varicellovirus* genus in the *Alphaherpesvirinae* subfamily of the *Herpesviridae* family, and is closely related to the human pathogens herpes simplex virus 1 (HSV-1) and varicella-zoster virus (VZV) (15). Similar to other alpha herpesviruses such as HSV-1, bovine herpesvirus 1 (BHV-1) and equine herpesvirus 1 (EHV-1), PRV exhibits marked neurotropism. The virus infects the mucosal epithelial cells and subsequently gains access to the peripheral nervous system (PNS), innervating the infected epithelium, which may even invade the central nervous system (CNS) *via* retrograde transport along the nervous system of its host (9). PRV replication in the CNS often results in nonsuppurative meningoencephalitis (10).

Suid herpesvirus type 1 is the alternative name for PRV, and the virus represents an important swine pathogen that leads to a devastating disease characterised by three main syndromes: 1) nervous system disorders and high mortality in young piglets; 2) respiratory disorders and growth retardation in finishing pigs; and 3) reproductive failure in sows (19). Although PRV can infect pigs of different ages, pigs exhibit an evident age-associated resistance to PRV, with up to 100% mortality in younger suckling piglets, less than 50% mortality in weaned piglets, and less than 5% in 5-month-old pigs. In addition to swine, PRV can also infect most mammals, including ruminants, carnivores, and rodents. Furthermore, a productive PRV infection in susceptible non-swine animals is invariably fatal and characterised by severe nervous signs. Only pigs are able to survive a productive PRV infection, subsequently becoming carriers of latent PRV infection established primarily in the trigeminal ganglia and tonsils. Piglets are highly susceptible to PRV infection, and only a low dose of PRV is needed to establish one. Dogs also appear to be highly susceptible, and there have been several reports of PRV infection in dogs, particularly in hunting dogs which acquired PRV while hunting wild animals (14). Moreover, it has recently

been reported that some commercial PRV vaccines are lethal to dogs (12). Although wild rats are thought to represent a source of PRV infection on swine farms and vector of the virus' spread between farms, the level of susceptibility of rats to PRV infection remains unclear.

PRV has a double-stranded linear DNA genome approximately 145 kb in length encoding more than 70 proteins. Some genes, *e.g.* gE, gI, gC, gG, UL23 (thymidine kinase, tk), US2, and US3 are nonessential for viral replication, but are related to viral virulence (13). Moreover, deletion of one or more of these genes results in an attenuated phenotype *in vivo* and a minor impact on the growth of cells cultured *in vitro*. However, it has also often been observed that the pathogenicity of some mutant PRV strains varied depending on the infected hosts. For example, the classical PRV vaccine strain, Bartha-K61, is avirulent in neonatal piglets, which are most susceptible to PRV infection, but can cause death in mice (11). In previous studies, PRV strains with tk deletion were avirulent in swine and mice, which indicates that tk represents a key virulence-associated gene. It is not clear whether tk-deleted PRV is avirulent in all susceptible animals. In this study, rats were infected with a tk-deleted PRV strain to assess the effect of tk deletion on PRV pathogenicity.

Material and Methods

Viruses and cells. The wild type PRV strain, JS-2012 (GenBank accession no. KP257591.1), was isolated from a diseased newborn piglet at a farm in Jiangsu province, China in 2012. The tk-deleted PRV strain SuHV-1 ΔTK:247 was derived from JS-2012 by deleting the sequences covering nucleotides (nt) 149 to 395 of the open reading frame (ORF) of the tk gene, as previously described (22). JS-2012 and SuHV-1 ΔTK:247 were propagated in Vero cells (ATCC CCL-81; ATCC, Manassas, VA, USA), which were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS) (Gibco) at 37°C with 5% CO_2 in a humidified incubator.

Rat infections. All rat experiments were performed in accordance with the protocols approved by the Animal Care and Ethics Committee of the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, under the number Shvri-rat-20190691. All protocols adhered to Guide for the Care and Use of Laboratory Animals (4).

To assess the virulence of SuHV-1 ΔTK:247 and JS-2012, 6-week-old Sprague Dawley rats were anaesthetised with an intraperitoneal injection of 0.5 mL 10% chloral hydrate. Six rats per group were inoculated intranasally with 20 μL (10 μL per nostril) or intramuscularly in a hind leg with 100 μL of different doses (10^5, 10^4, 10^3, 10^2, or 10 $TCID_{50}$). The infected rats

were monitored for 3 weeks. All surviving rats were euthanised at the end of the experiments and those that died during the experiment were immediately necropsied. Various tissues, including the trigeminal ganglion, olfactory bulb, cerebrum, cerebellum, spinal cord, lung, spleen, liver, kidney, and bladder from all rats were collected and stored at −80°C.

Viral DNA extraction and real-time PCR assay. Portions (50 mg) of the collected tissues were placed in a 2 mL microcentrifuge tube containing sterile steel beads and 250 μL of phosphate-buffered saline (PBS), and subsequently homogenised using a TissueLyser (Ningbo Scientz Biotechnology Co., Ningbo, China). The homogenised tissues underwent three freeze-thaw cycles and were finally centrifuged at $10,000 \times g$ for 3 min at 4°C. DNA was extracted from 200 μL of supernatant with a TIANamp Virus DNA/RNA Kit (Tiangen Biotechnology Co., Beijing, China) according to the manufacturer's instructions, and finally eluted in 50 μL of buffer. Genomic DNA of PRV was quantified using a verified real-time PCR assay to amplify the PRV gB genes with the following primers: forward gB505F, 5′-AAGTTCAAGGCCCACATCTA-3′; reverse gB592R, 5′-TGAAGCGGTTCGTGATGG-3′; and probe 5′-FAMCAAGAACGTCATCGTCACGACCG-BHQ-3′, as previously described (16).

Statistical analysis Data were analysed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). All data are presented as the mean ± standard deviation (SD). A P value of < 0.05 was considered statistically significant.

Results

To evaluate the pathogenicity of SuHV-1 ΔTK:247 and JS-2012, 6-week-old SD rats were infected *via* intranasal or intramuscular inoculation. As shown in Fig. 1, rats inoculated intranasally with 10^5, 10^4, and 10^3 $TCID_{50}$ SuHV-1 ΔTK:247 displayed 100% (6/6), 83.3% (5/6), and 50% (3/6) mortality, respectively. The LD_{50} value of SuHV-1 ΔTK:247 was $10^{3.16}$ $TCID_{50}$. However, the pathogenicity of SuHV-1 ΔTK:247 in rats following intramuscular inoculation required a high dose. Only an infection at 10^5 $TCID_{50}$ caused death in rats with 100% mortality, whereas other rats infected with less than 10^5 $TCID_{50}$ survived. Compared to SuHV-1 ΔTK:247, JS-2012 displayed increased pathogenicity to rats following both intranasal and intramuscular inoculation. The LD_{50} values of JS-2012 *via* intranasal or intramuscular inoculation were $10^{2.4}$ and $10^{2.84}$ $TCID_{50}$, respectively. Before death, all rats showed pruritus in the nostrils or the skin around the injection sites. These results indicated that tk-deleted PRV was highly pathogenic to rats, particularly *via* intranasal inoculation; however, deletion of the tk gene could also diminish PRV pathogenicity to rats to a certain degree, especially *via* intramuscular inoculation.
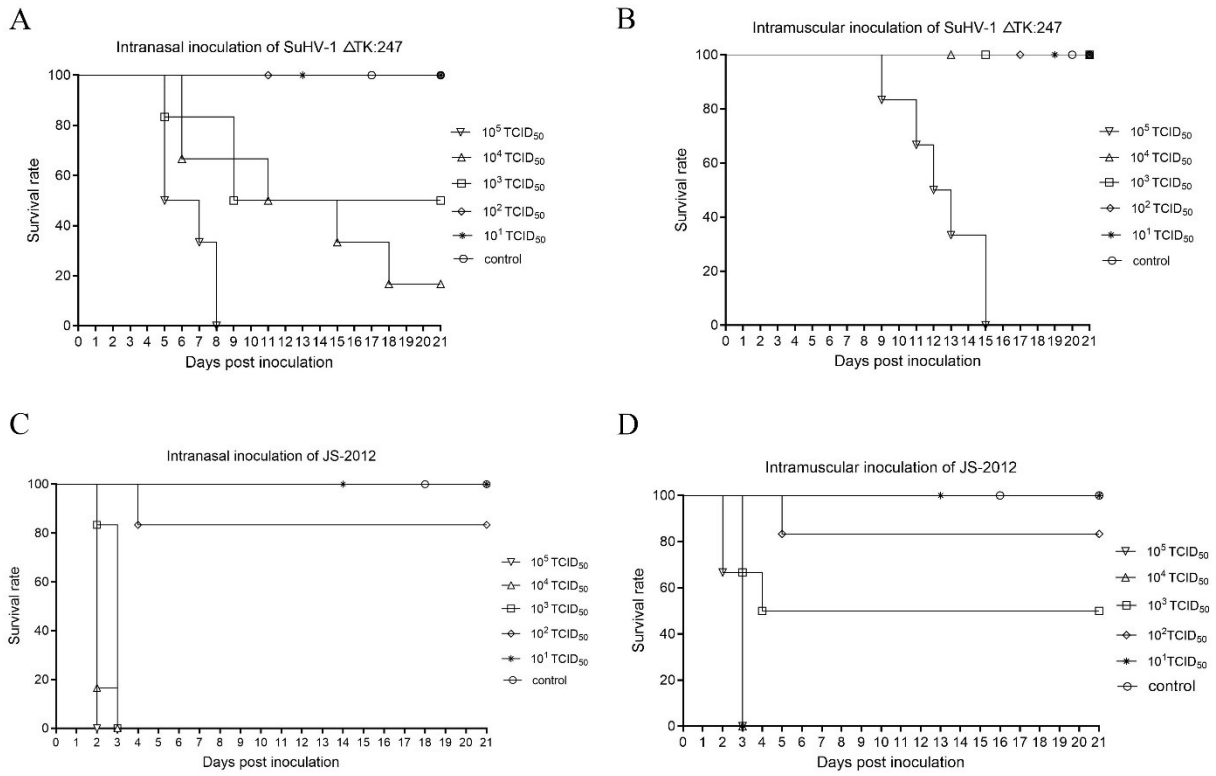
Fig. 1. The survival rate of the rats infected with PRV JS-2012 or SuHV-1 ΔTK:247. The percentage of surviving rats is presented as a Kaplan-Meier plot (n = 6 per group)
A - rats intranasally inoculated with the tk-deleted PRV strain, SuHV-1 ΔTK:247; B - rats intramuscularly inoculated with SuHV-1 ΔTK:247; C - rats intranasally inoculated with the wild-type PRV strain, JS-2012; D - rats intramuscularly inoculated with JS-2012
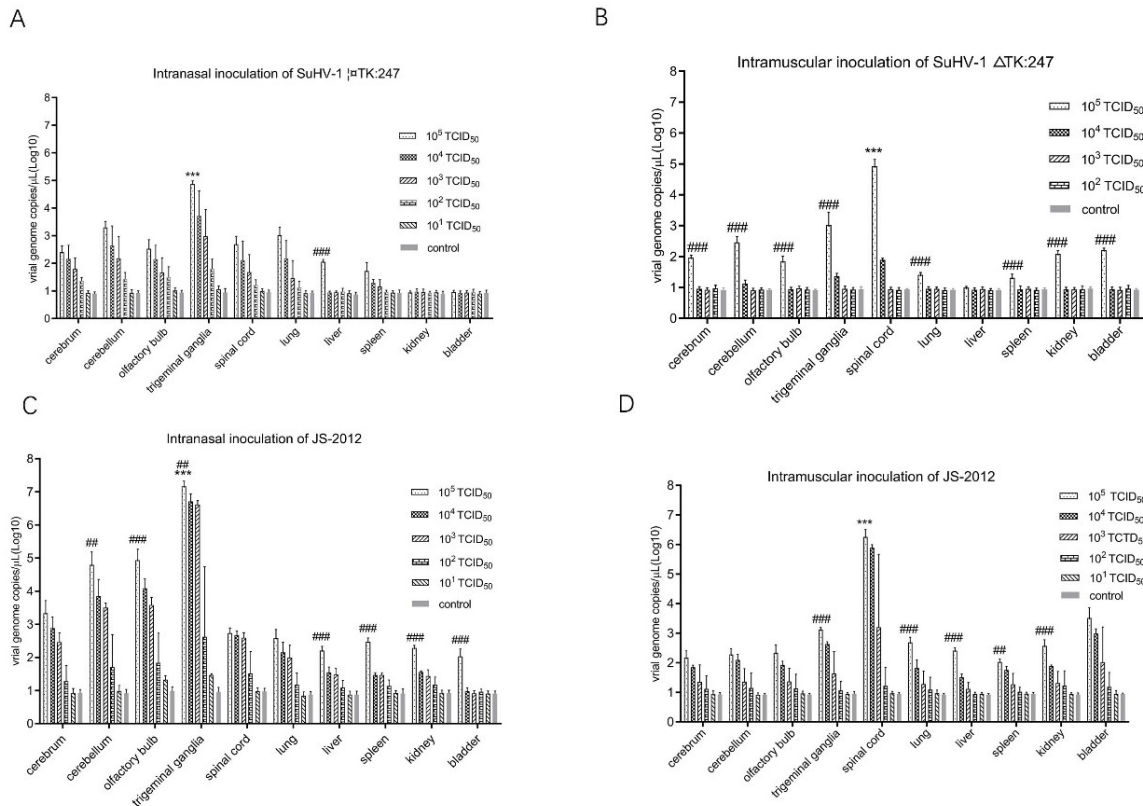

Fig. 2. The viral loads in different tissues of rats infected with PRV JS-2012 or SuHV-1ΔTK:247, respectively. The rats in each group (n = 6 per group) were infected with JS-2012 and SuHV-1 ΔTK:247 at different doses *via* intranasal or intramuscular inoculation. Ten tissues were collected from dead and surviving rats, and subjected to viral load analysis. Viral copies were determined by qPCR. Data are presented as the mean ± SD from six rats per group. *** - P < 0.001 *versus* other tissues of rats infected with 10^5 $TCID_{50}$ PRV; ### - P < 0.001 and ## - P < 0.01 *versus* the same tissues of rats infected with other doses of PRV

Ten tissues (cerebrum, cerebellum, olfactory bulb, trigeminal ganglia, spinal cord, lung, spleen, liver, kidney, and bladder) of dead and euthanised rats were collected to determine the viral loads. As shown in Fig. 2, the major tissues infected by JS-2012 and SuHV-1 ΔTK:247 consisted of the nervous tissues, including the trigeminal ganglion, spinal cord, cerebrum, cerebellum, and olfactory bulb. However, the tissue with the highest viral load varied depending on the inoculation route. The highest viral load following intranasal inoculation appeared in the trigeminal ganglia, whereas the spinal cord exhibited the highest titre following intramuscular injection. When infected *via* the same route, the level of JS-2012 viral load was higher than that of the SuHV-1 ΔTK:247 loads in the nervous tissues of dead rats. The JS-2012 loads in the trigeminal ganglia could reach 10^7 copies/μg through intranasal inoculation, and those in the spinal cord reached 10^6 copies/μg through intramuscular inoculation, while the SuHV-1 ΔTK:247 loads in the trigeminal ganglia did not exceed 10^5 copies/μg following intranasal inoculation, and those in the spinal cord had maxima of approximately 10^5 copies/μg following intramuscular inoculation. Besides accumulating in the nervous tissues, JS-2012 tended to disseminate to some parenchymatous organs, including the spleen, liver, and kidney through both the intranasal and intramuscular infection routes, although the JS-2012 viral load was low in these organs. In contrast, SuHV-1 ΔTK:247 appeared to be limited regarding the spread within nervous tissues. With the exception of some SuHV-1 ΔTK:247 found in the spleen, liver or kidneys of the dead rats infected with the highest dose of 10^5 TCID$_{50}$, low viral loads were identified in these tissues in SuHV-1 ΔTK:247-infected groups. In addition, the viral loads in the tissues of the surviving rats were very low, which resulted in large SD values in some of the rat groups.

Discussion

Productive PRV infection in rodents is invariably fatal; however, deletion of tk could attenuate PRV and result in a loss of lethality to mice. In our previous study, the SuHV-1 ΔTK:247 tk-deleted strain was confirmed to have lost thymidine kinase activity, and BALB/c mice intranasally infected with 10^4 or even 10^5 TCID$_{50}$ SuHV-1 ΔTK:247 neither showed clinical signs nor died (22). In this study, we found that SuHV-1 ΔTK:247 retains high pathogenesis in rats inoculated intranasally and the LD$_{50}$ value of SuHV-1 ΔTK:247 was $10^{3.16}$ TCID$_{50}$. These results indicated that the pathogenicity of tk-deleted PRV to different kinds of rodents varied to a large extent.

Thymidine kinase is involved in nucleoside metabolism across a broad range of eukaryotes, prokaryotes, and even some DNA viruses, including herpesviruses. Compared to cellular tks, which are specialised enzymes catalysing the transfer of the γ-phosphate of ATP to 2′-deoxythymidine in the nucleoside salvage pathway, tks encoded by alpha herpesviruses share broader substrate specificity. Moreover, tks can phosphorylate both deoxythymidine and deoxycytidine, as well as a series of nucleoside analogues, *e.g.* acyclovir (21). Thus, controlling cellular nucleotide metabolism may represent an important pathway for host cells to limit DNA virus infection. The host protein SAMHD1, which possesses dNTP triphosphohydrolase activity, has been shown to restrict DNA viruses including HSV-1 by limiting DNA replication (5, 6). In addition, tk deletion often attenuates alpha herpesviruses infection in susceptible animals, although it has limited effects on viral growth in host cells *in vitro*. This indicates that tk supplies dNTP substrates essential for the viral DNA polymerase and plays a crucial role in alpha herpesvirus infection (3, 8, 18). In general, the tk gene and/or gE/gI genes have been deleted to develop PRV vaccines (2). The first genetically engineered live PRV vaccine strain was derived from the Bucharest strain by deleting only the tk gene (7). Although the gE/gI-deleted PRV strains were avirulent in piglets, they could cause death in both mice and sheep (1). When ten putative virulence genes were knocked out of a virulent PRV variant, only the tk-deleted virus completely lost its pathogenicity in mice (17). Thus, the tk gene appeared to be the primary virulence factor for PRV. However, it was clearly demonstrated that the tk-deleted PRV retained relatively high pathogenicity in rats following intranasal inoculation. The experimental SuHV-1 ΔTK:247 strain could enter the CNS of infected rats and the infected rats showed pruritus before death. These results suggested that rats represented a unique host species for PRV infection.

In this study, wild-type PRV exhibited high pathogenicity in rats. The LD$_{50}$ values of PRV JS-2012 in rats were $10^{2.4}$ and $10^{2.84}$ TCID$_{50}$ *via* intranasal and intramuscular inoculation, respectively. The susceptibility of rats to PRV differed from that of a previous report, in which a rather high dose of PRV (> 10^4–10^5 TCID$_{50}$) was commonly necessary to infect animals apart from piglets (19). In the study by Wei *et al.* (20), 60% mortality was inflicted on C57BL/6J mice intramuscularly infected with 5×10^4 TCID$_{50}$ of the PRV strain Min-A. Considering that tk-deleted JS-2012 was highly pathogenic to rats, rats should be highly susceptible to PRV infection. Thus, rats may represent an important vector for the spread of PRV from domestic pigs to cats, dogs, and wild carnivores. Moreover, cats and dogs may be naturally infected with PRV (23), and some commercial PRV vaccines are lethal to dogs (12).

In summary, rats were highly susceptible to PRV infection, and tk-deleted PRV retained relatively high pathogenicity in rats inoculated intranasally. However, tk deletion also reduced PRV virulence to a certain degree in rats. Compared to wild-type PRV, the virulence of tk-deleted PRV *via* intramuscular inoculation was markedly declined.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Animal Rights Statement: All rat experiments were performed according to the Guide for the Care and Use of Laboratory Animals of the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China. The animal ethics approval number was Shvri-rat-20190691.

*These authors contributed equally to this work.

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