TK/gI/gE/11K Genes Is Not Involved in the Virulence Enhancement of Highly Virulent Pseudorabies Virus

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Research Article

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Abstract

The large-scale outbreaks of Pseudorabies in china since 2011 in vaccine-immunized pig farms were caused by the highly virulent Pseudorabies virus (PRV). To investigate the factors involved in the virulence enhancement of the variant PRV, four recombinant viruses with the virulence gene's replacement, *gI/gE/11K and TK/gI/gE/11K*, between the variant strain HN1201 and the classical strain Fa were constructed based on bacterial artificial chromosome infectious clones. Compared with their parental strain, the viral titers of the recombinant PRV strains are not strongly influenced by the replacement of *TK/gI/gE/11K*, while as previously reported, the strain HN1201 and its derived viruses showed the higher mortality, the severer clinical symptoms and tissues damage than that of strain Fa and its derived strains. In summary, the *TK/gI/gE/11K* genes of variant strain HN1201 showed no contribute to its virulence enhancement compares with the classical strain.

Introduction

Pseudorabies, also known as Aujeszky's disease, caused by Pseudorabies virus (PRV), is a devastating disease in pigs and leads to significant economic losses worldwide. PRV, a linear, double-stranded DNA virus, is an Alphaherpesvirinae subfamily member of the Herpesviridae family, within the order Herpesvirales [18], and with genome length of about 150 kb, containing at least 72 open reading frames [10], which infected most of the domestic and wild animals, characterized by fever, nervous disorder accompanying frequently by intense pruritus itching and encephalomyelitis. In pigs, the clinical symptom varies depending on the age, the infection of piglets is characterized by central nervous system disorders and death, and in older pigs is the respiratory symptom or reproductive failure [19]. Like other alphaherpesviruses, PRV infection showed a lifelong latent in the peripheral nervous systems and could be reactivated by stress factors or immunosuppression effect, which increased the difficulty for Pseudorabies prevention and control [15].

Vaccination is the pivotal strategy for Pseudorabies control. Pseudorabies had been eliminated in many countries by employing the most widely used vaccine Bartha-K61 [13, 14], and which also had played a critical role in the control of Pseudorabies in China. While the mass vaccination confers the protection of classical virus infection but not for the wild-type virus [31]. Especially in late 2011, severe outbreaks of Pseudorabies occurred on several pig farms with Bartha-K61 vaccination in China, the infected pigs exhibited high fever (> 40°C), anorexia, coughing, dyspnea and systemic neurological symptoms [1, 28, 31]. Later, the disease spread to most provinces in China. The highly virulent PRV was proved to be the causal agent of this epidemic [1, 6, 28, 31].

PRV strains evolved in the outbreaks were isolated and the complete genomic sequences were used for comparative studies between the classical and vaccine strains [13, 22, 25, 26]. The first reported PRV virulent wild-type strain HN1201, isolated from an affected farm in 2012, showed more severe clinical symptoms and pathological lesions than that of classical strain Fa, and with more abundant antigen distribution in extensive organs [28, 29].

Bacterial artificial chromosome (BAC) is wildly used for the studies of viral gene function and viral genome manipulation in the form of BAC infectious clone, like herpesvirus [24]. The first full length BAC clone of PRV was established in 1999 for investigating the function of individual viral genes [20].This technique enables mutagenesis of the viral genome using the bacterial recombination machinery.

BAC clone of PRV strain HN1201 was constructed for the generation of gene deletion vaccine candidates, or the studies of pathogenesis and immune mechanism [32]. In this study, to further exploring the pathogenicity enhancement mechanism, the infectious BAC clone of classical PRV strain Fa was constructed. The major virulence-associated genes, *gI/ gE/ 11K* and *TK/gI/gE/11K*, were replaced between these two infectious clones, and the pathogenesis of these chimeric viruses was evaluated.

Material And Methods

Animals

Pigs (n = 35) used in this study were tested negative for PRV gB and gE antibodies by commercial ELISA kits (gB: Pseudorabies Virus gB Antibody Test Kit, IDEXX, USA; gE: Pseudorabies Virus gpI Antibody Test Kit, IDEXX, USA).

Generation of BAC clone

Previously, a BAC plasmid pBAC-HN1201^{TK-} containing the genome of virulent strain HN1201 (GenBank: KP722022) with *TK* gene deletion was constructed [32]. Similarly, the infectious BAC clone of classical strain Fa (purchased from the Institute of China Veterinary Medicine Inspection) [29] with *TK* gene deletion, pBAC-Fa^{TK-}, was constructed according to the strategy as pBAC-HN1201^{TK-} construction.

To construct PRV BAC clones with Fa *gI/gE/11K* or *TK/gI/gE/11K* genes replacement in HN1201genome, the plasmid pBAC-HN1201^{TK-} was used as the backbone. Firstly, a PCR fragment containing an I-*Sce*I site together with the ampicillin (Amp) gene flanked by a 50 bp duplicated sequence of HN1201 was amplified, and then be electroporated into the DY380 competent cells harboring pBAC-HN1201^{TK-} [21, 24] to obtain the positive clones of pBAC-HN1201$^{TK-/gI-/gE-/11k-}$Amp, which were confirmed by digestion of *Bam*HI. After that, a PCR fragment containing an I-*Sce*I site together with the kanamycin (Kan) gene and the gI/gE/11k genes of Fa strain flanked by a 500 bp sequence of HN1201 in each end was amplified, and then be transformed into *E. coli* DY380 competent cells to obtain the intermediate plasmid pBAC-HN1201^{TK-}-Fa$^{gI+/gE+/11K+}$-Kan. After digested with I-*Sce*I, the linear plasmid was transformed into DY380 cells to remove the Kan gene, and the bacterial strain of pBAC-HN1201^{TK-}-Fa$^{gI+/gE+/11K+}$ was obtained. A PCR fragment containing the Cat-OriS sequence (from pBeloBAC11), I-*Sce*I site, Kan gene, *TK* gene and a 850 bp flanking sequence from HN1201 genome was amplified, and then be transformed into pBAC-HN1201^{TK-}-Fa$^{gI+/gE+/11K+}$ competent cells to construct the intermediate plasmid pBAC-HN1201^{TK+}-Fa$^{gI+/gE+/11K+}$-Kan with the reinsertion of HN1201 *TK* gene, after digested with I-*Sce*I, the plasmid pBAC-

HN1201^{TK+}-Fa$^{gI+/gE+/11K+}$ (Fig. 1) was constructed after removing the Kan gene by linear plasmid transformation into *E. coli* DY380.

The plasmids pBAC-HN1201-Fa$^{TK+/gI+/gE+/11K+}$, pBAC-Fa^{TK+}-HN1201$^{gI+/gE+/11K+}$ and pBAC-Fa-HN1201$^{TK+/gI+/gE+/11K+}$ (Fig. 1) were constructed according to the procedure described as pBAC-HN1201^{TK+}-Fa$^{gI+/gE+/11K+}$ construction.

Recombinant viruses rescue

The recombinant PRV was rescued by transfecting pBAC plasmid into Vero cells as illustrated previously [32], the GFP gene and BAC gene cassette were deleted after cell passaging. After purification by plaque assay, the virus rescued from pBAC-HN1201^{TK+}-Fa$^{gI+/gE+/11K+}$, pBAC-HN1201-Fa$^{TK+/gI+/gE+/11K+}$, pBAC-Fa^{TK+}-HN1201$^{gI+/gE+/11K+}$ and pBAC-Fa-HN1201$^{TK+/gI+/gE+/11K+}$ were named as HN1201^{TK+}-Fa$^{gI+/gE+/11K+}$, HN1201-Fa$^{TK+/gI+/gE+/11K+}$, Fa^{TK+}-HN1201$^{gI+/gE+/11K+}$, and Fa-HN1201$^{TK+/gI+/gE+/11K+}$, respectively.

In vitro growth properties and plaque morphology

Multiple growth curves of rescued viruses were assessed and compared with those the parental virus HN1201 and Fa. ST cell monolayer was infected with virus at an MOI of 0.001, and then the supernatants were harvested at 12, 24, 36, 48, 60, 72, 84 and 96 hour-post-infection (hpi). The virus titers were determined using a microtitration infectivity assay and calculated as $TCID_{50}$/mL using the Reed-Muench method. The test is triplicate repeated at each time point. Plaque morphology and sizes were determined at 80 hpi on swine testis (ST) cells. After incubation for 1 hour, the medium was discarded, and cells were overlaid with Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, USA) containing 1% low-melting point agarose and 2% fetal bovine serum (Gibco). For each virus, 100 plaques were randomly selected, and their size determined by ImageJ software. The plaque size of HN1201 was set at 100%.

Pathogenicity analyses of BAC infectious clone virus and the chimeric recombinant viruses

Thirty 35-day old pigs were randomly divided into 7 groups (n = 5), all the pigs were inoculated by intranasally with $10^{7.0}$ $TCID_{50}$/mL virus per pig excepted the group 7 (unchallenged control). Pigs in group 1 to 6 were challenged with HN1201, HN1201^{TK+}-Fa$^{gI+/gE+/11K+}$, HN1201-Fa$^{TK+/gI+/gE+/11K+}$, Fa, Fa^{TK+}-HN1201$^{gI+/gE+/11K+}$, and Fa-HN1201$^{TK+/gI+/gE+/11K+}$, respectively. The body temperature and the clinical signs were recorded daily. Pigs showed clinical symptoms and dying within 14 days were executed and necropsied. After 14 days, all surviving pigs were euthanized and necropsied, the samples of tonsil, lung, brain, trigeminal ganglion were collected for immunohistochemistry (IHC) assay.

IHC assay

The collected samples were fixed with 10% neutral-buffered formalin for IHC assay as descried previously [29, 32]. The primary antibody used in IHC assay was the PRV monoclonal antibody 3B5, and the HRP goat anti-mouse IgG (BTI, United States) was served as the secondary antibody. After staining with

hematoxylin and washing, the slides were allowed to dry naturally and mounted with water-soluble tablets seal before visualizing by 200× microscope photographs. According to the intensity of stains, the results were determined by negative (-) or positive (+).

Results

Construction of gI/gE/11K and TK/gI/gE/11K chimeric recombinant viruses between PRV HN1201 and Fa

At 72 h post-transfection of pBAC plasmids in Vero cells, the obvious visible cytopathic effect could be observed (Fig. 2). The gene replacement of *gI/gE/11K* or *TK/gI/gE/11K* in the four rescued viruses were identified by sequencing (data not shown), and then be subjected to serial passage in ST cells.

In vitro growth properties of the chimeric viruses

The virus titers in passages 5 were determined. As shown in Fig. 3a, the viral titers of the recombinant PRV viruses are not strongly influenced by the replacement of *TK/ gI/ gE/ 11K* between HN1201 and Fa, all with the peak titer at 60–72 hpi. Plaques formed by chimeric variants HN1201^{TK+}-Fa$^{gI+/gE+/11K+}$ and HN1201-Fa$^{TK+/gI+/gE+/11K+}$ were smaller than that formed by their parental virus HN1201, but with no difference between these two chimeric variants. As shown in Fig. 3b and 3c, similar phenomenon in Fa strain and its chimeric variants could be observed.

Pathogenesis differences of recombinant viruses to piglets

All the pigs inoculated with HN1201 in group 1 exhibited high fever, depression, anorexia, cough, and systematic neurological signs like convulsion and ataxia, and all died at 5–6 dpi. As with the clinical signs of the HN1201-inoculated group at 2–7 dpi, pigs challenged with HN1201^{TK+}-Fa$^{gI+/gE+/11K+}$ and HN1201-Fa$^{TK+/gI+/gE+/11K+}$ in group 2 and 3 also showed typical clinical manifestations and died at 5–7 dpi (Fig. 4a). The pigs body temperature in HN1201^{TK+}-Fa$^{gI+/gE+/11K+}$ challenge group was lower than that of HN1201 challenge group, while was slightly higher than that of HN1201-Fa$^{TK+/gI+/gE+/11K+}$ challenge group (Fig. 4b).

In contrast, pigs challenged with Fa in group 4 exhibited high fever, depressed spirit, decreased appetite, and no pigs displayed neurological symptoms, which was much milder than that of HN1201, and all pigs recovered from 7 dpi. As shown in Fig. 4c, only minimal changes of temperature could be found among groups 4, 5 and 6 for all time points. In the groups 5 and 6, except for depressed spirit and decreased appetite, only two of five pigs in both groups showed convulsion, shiver, and salivation. Generally, the clinical symptoms of pigs challenged with Fa and its derived virus were much slighter than these of HN1201 and its derived virus.

IHC assay

Tonsil, lung, brain and trigeminal ganglion samples collected from pigs were used for the IHC assay. As shown in Fig. 5, compare to the pigs in the unchallenged group, the positive reaction of groups 2 and 3 was weaker than that in group 1 with HN1201 challenge, especially in trigeminal ganglion. For Fa related groups, four of five pigs in group 5 showed the most serious lung lesions, but the neuropathy (including convulsion and ataxia) in group 6 was weaker than that in groups 4 and 5, which was consistent with the clinical symptoms in group 5 (two of five pigs exhibited neurological symptoms), but no pigs showed neurological symptoms in group 6.

Discussion

Pseudorabies has caused a huge impact on world pig industry since in the early 20th century. The use of vaccines Bartha-K61 allowed the well control of Pseudorabies in many countries including China. However, since 2011, an increased number of immunized farms had pseudorabies outbreaks, and multiple several variant PRV had been isolated [1, 6, 7, 13, 25, 27, 28, 30, 31]. These findings suggest that pigs are not fully protected due to the enhancement of the pathogenicity of these variant PRV. Compare with the classical wild type strain or vaccine strain, the genome-wide variations were found in the variant strain [13, 27, 30], most of the glycoproteins contributing to the adsorption, immunity, or virulence have undergone large variations [4, 27, 30].

TK gene located in the UL had been shown to be a major neurovirulence determinant for pseudorabies virus [23], which was first reported to be associated with virulence of PRV because of the attenuated property of TK^- strains for pigs, and the glycoproteins *gE* and *gI* affect the biological characteristics of PRV in the same way in the form of noncovalently complex [33]. It was proved that *gE* is more important than *gI* in the neural invasion and the spread of PRV [11, 16]. The deletions of *gI*, *gE*, *11k* and *28k* of short unique regions (Us) in vaccine strain Bartha genome contribute to its decreased virulence, although is not the only factor [9, 12, 17]. Other glycoproteins and enzymes involved in virus replication also contribute to the PRV virulence [2, 5, 8]. Genome-wide sequence analysis demonstrated that almost all the viral proteins displayed variations in the highly virulent PRV strain [13], which makes it is more difficult to find genes associated with increased virulence. The PRV with deletion of *TK*, *gI*, *gE* and *11k* was severely attenuated and had no pathogenicity to pigs (data not shown), which showed that the four main virulence genes were directly related to virus pathogenicity. So, *gI*, *gE* and *11K* substitution with or without *TK* gene was evaluated simultaneously in this study.

By replacing *gE* and *gI* gene between virulent strain ZJ01 and Chinese prototypic strain LA, rZJ01-LA/gEI retained high virulence in piglets except for the obviously prolonged survival time, whereas rLA-ZJ01/gEI showed higher virulence than its parental LA, but overall, ZJ01 and its derivates were still more pathogenic than LA and its derivates [3]. In this study, similar result could be observed when the pathogenicity was evaluated between the virulence gene chimeric strains and the parental strains. rHN1201 harboring *gI/ gE/ 11K* or *TK/ gI/ gE/ 11K* virulence genes of Fa exhibited much higher mortality and more severe clinical signs than rFa, only a slight difference of IHC assay between the recombinant strains and their parental viruses, displayed a weaker staining than parental strain HN1201. While for Fa

and its recombinant strains, the staining of the nervous system and lung tissue is irregular. In spite of this, the virulence reduction of the strain HN1201 with *TK/ gI/ gE/ 11K* replacement was still significantly lower than that of the two-gene replacement strain of the ZJ01, this may be partly due to the virulence difference between classical strain Fa and LA. But more likely, the variation in major virulence genes may be not the main cause of increased virulence in the pandemic strain. For such a large genome of PRV, the variation of other virulence genes, and the interaction between non-virulence and major virulence genes may play an important role in the pathogenetic enhancement.

In summary, the *TK/gI/gE/11K* genes of variant strain HN1201 did not contribute to its virulence enhancement compared with the classical strain Fa. The deep learning based analysis in the viral genome for revealing PRV virulence enhancement needs further investigation.

Declarations

Acknowledgements

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Ethics approval

The animal expreiments were approved by the Animal Care and Ethics Committee of National Research Center for Veterinary Medicine (Permit 20180225001).

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

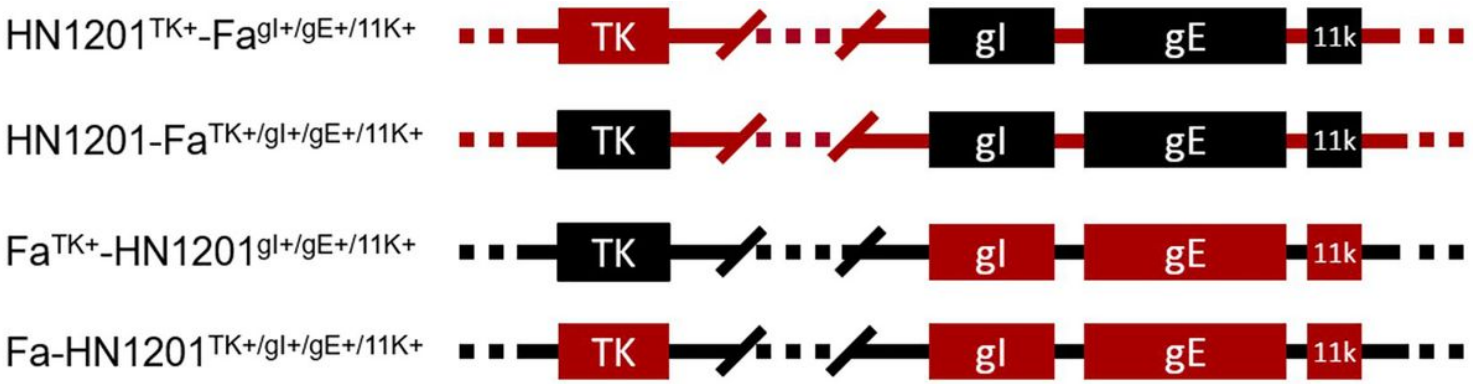

Figure 1

Structure of chimeric viruses. The HN1201 and Fa genomes were shown in red and black, respectively.

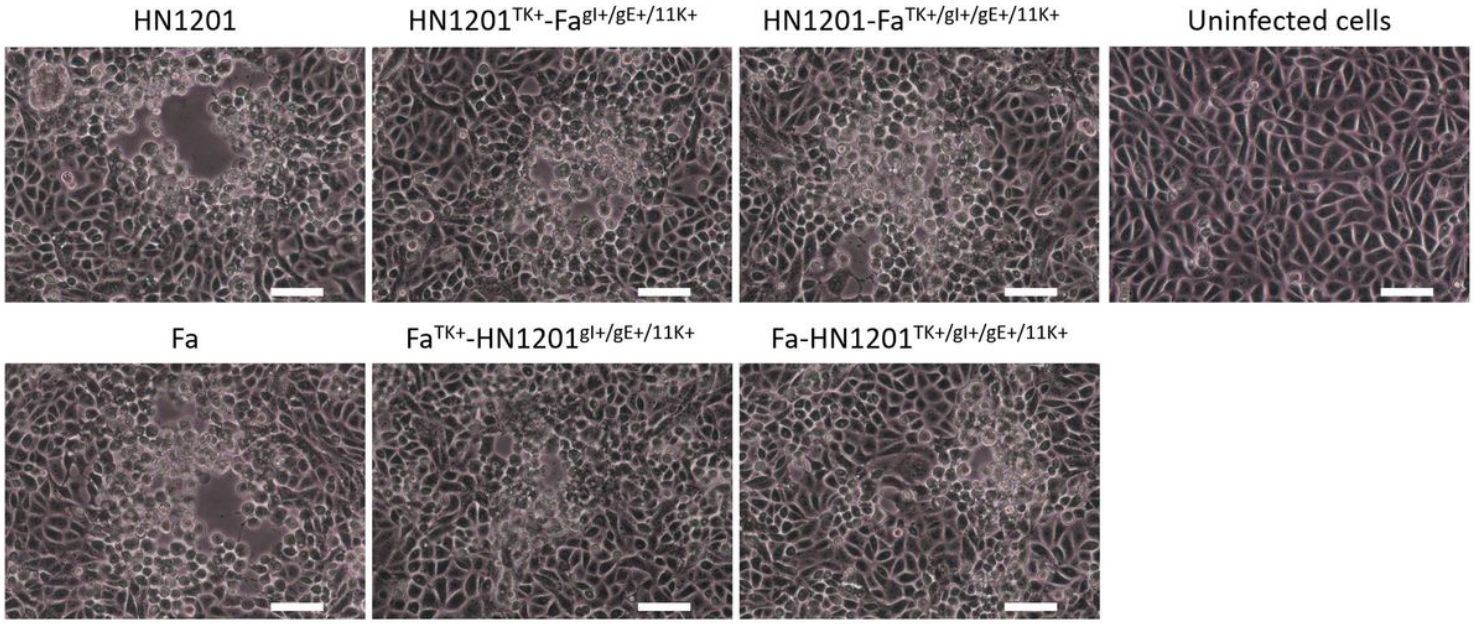

Figure 2

Cytopathic effect in Vero cells after transfection with recombinant PRV pBAC plasmids. Bar = 50 μm.

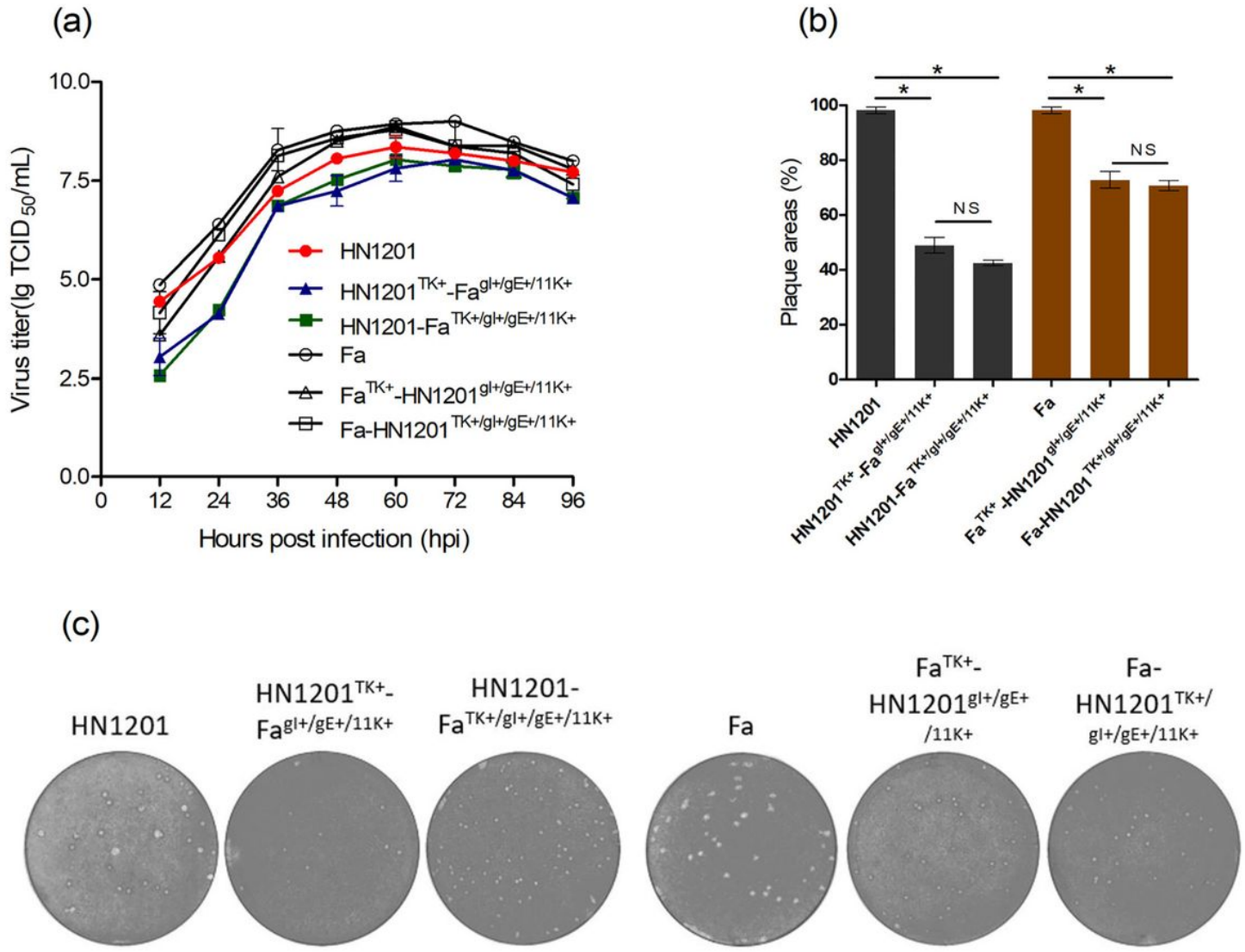

Figure 3

Multiple growth curves and plaque assay of chimeric and parental viruses. (a) Multiple growth curves of the chimeric viruses. ST cells grown in a 6-well plate were infected with parental or recombinant HN1201 and Fa at 0.001 MOI. The culture supernatants were collected at the indicated time points for the viral titer determination. (b, c) Plaque morphology and plaque size of the recombinant viruses. ST cells grown in a 6-well plate were infected with parental or recombinant HN1201 and Fa. The plaque size induced by parental virus were set at 100%. Asterisk denotes a statistically significant difference ($P < 0.05$). NS indicates no statistical difference.

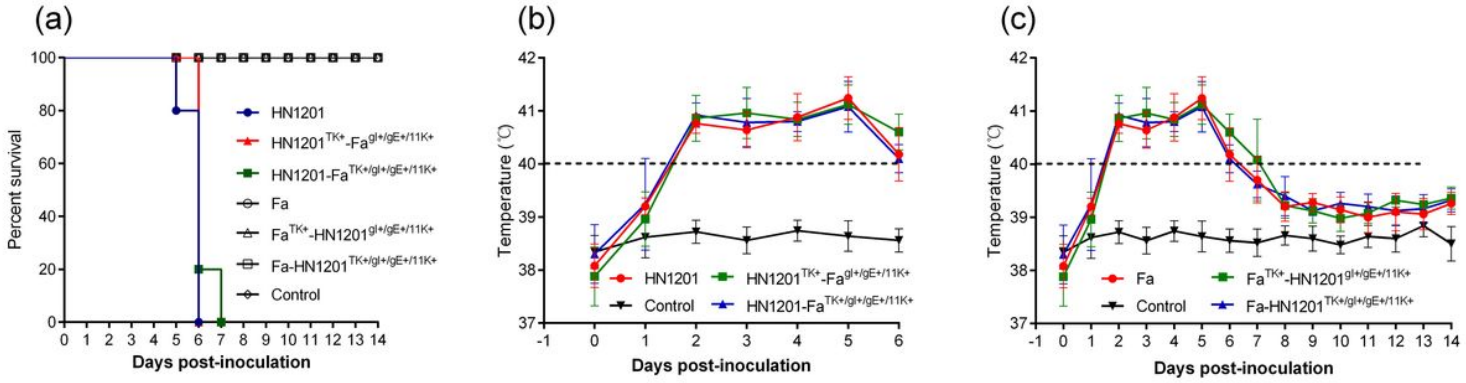

Figure 4

Survival rate and body temperature of pigs after challenging with recombinant and parental viruses. (a) Survival rate of pigs challenged with HN1201, Fa and their derived viruses. (b) Body temperature of pigs challenged with HN1201, HN1201TK+-Fagl+/gE+/11K+ and HN1201-FaTK+/gl+/gE+/11K+. (c) Body temperature of pigs challenged with Fa, FaTK+-HN1201gl+/gE+/11K+ and Fa-HN1201TK+/gl+/gE+/11K+.

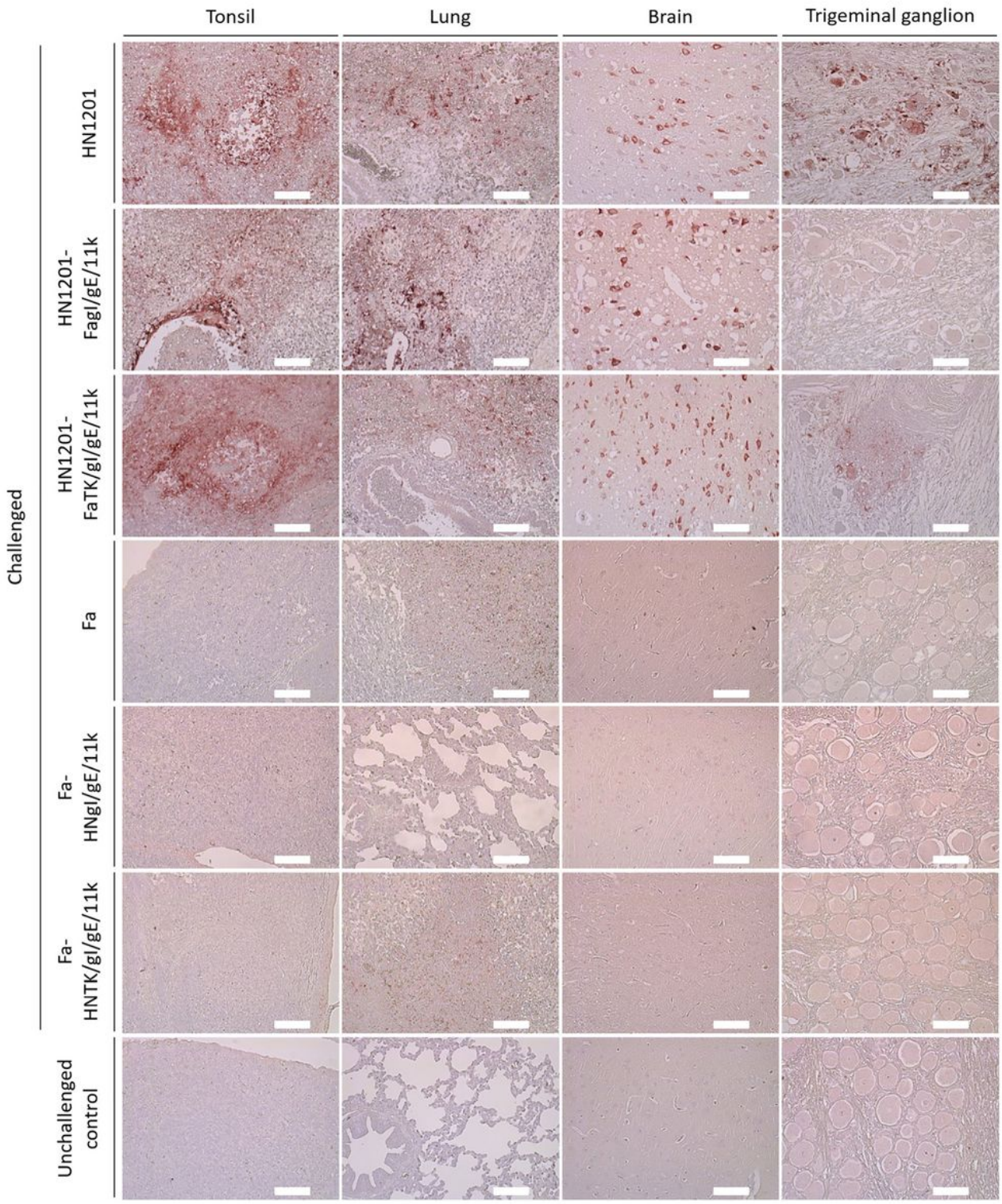

Figure 5

Results of IHC assay of tonsils, lungs, brains, and trigeminal ganglions. Representative IHC images of tonsils, lungs, brains, and trigeminal ganglions corresponding to animal groups infected with the viruses indicated on the left. Bar = 50 μm.