RESEARCH ARTICLE

Distribution of Pseudorabies Virus in Kunming Strain Mouse Via Gavage, Intranasal and Subcutaneous Infection

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ABSTRACT

The emergence of variant pseudorabies virus caused great economic losses in domestic farms in the recent past. The mice were infected with Halv strain PRV via intragastric administration, intranasal and subcutaneous into the cervical skin to study the Pseudorabies in mouse model. The gross lesions, microscopic lesions and the distribution of PRV in mice were investigated. All the mice in each group showed exciting and roughening of the fur and frequent scratching. Microscopic lesions included mild-to-severe interstitial pneumonia, hemorrhage and nonsuppurative encephalitis in brain. The distribution of PRV in three groups was different. In intragastric administration group, PRV antigens were distributed in brain hippocampus, medulla oblongata, gray substance spinal cord and intestinal submucosa. In intranasal group, PRV antigens were detected in gray substance spinal cord, pons, myocardial cells, pulmonary epithelial cells and lymphocytes in red pulp of the spleen. In subcutaneous group, the PRV antigens were located in the sensory and motor cortex, prefrontal cortex, gray matter in spinal cord, purkinje cells in the cerebellum and lymphocytes in the splenic red pulp. These findings provided evidence that the distribution of PRV was depended on injected-routes.

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INTRODUCTION

Pseudorabies virus (PRV) is a dsDNA virus belongs to Herpesviridas, subfamily Alphaherpesvirinae, Suid herpesvirus-1. PRV is endemic around the world and causes severe economic losses (Wu *et al*., 2013; Zhang *et al*., 2015). Swine is PRV's natural host, but there are numerous secondary hosts, which include almost all the mammals. PRV can infect piglets and result in shivering and opisthotonos. The mortality of PRV infection might reach 100%. In the second hosts, the clinical signs are mainly pruritus which frequent leads to death after few hours (Wozniakowski and Samorek-Salamonowicz, 2015). Through the efficient vaccination, PRV had been well controlled in swine industry worldwide including the United States and some countries in Europe (An *et al*., 2013). However, variant PRV strains emerged from vaccinated pigs with a newly virus antigenic in recent years. The variant PRV always related to the immunity failure when commercial PRV vaccines were used.

PRV infection was usually via intranasal and oral routes, and intranasal route is particular common (Babic *et al*., 1996). PRV initially replicate in the epithelial cells, and then go through mucous layer, access the submucosa and enter into blood vessels and nerve endings. Finally, the particles may spread throughout the internal organs (Nauwynck and Pensaert, 1995; Glorieux *et al*., 2011; Yang *et al*., 2012). But virusemia in PRV infection is still controversial (Wittmann, Jakubik and Ahl, 1980). When PRV accessed to the nerve endings, it retrograde spread to the first-order neurons, and continues to second-order and third-order neurons in the central nervous system (CNS) through axonal, and then anterograde spread to corresponding innervation organs (Lyman *et al*., 2008; Lee *et al*., 2011). PRV could establish a latent infection and the host had no clinical sign. It could be an infectious source to other animals (Cheung, 1989). When PRV infection occurred in the stomach wall it could transport transneuronally via vagal and sympathetic to the medullary dorsal vagal complex and thoracic dorsal horn, then transported into pontine reticular formation and CNS (Rinaman and Schwartz, 2004). PRV often propagated to neurons through trigeminal, sympathetic, parasympathetic and olfactory pathways (Babic *et al*., 1996). In swine,

trigeminal and olfactory pathways were the most common. Whereas in mouse, the parasympathetic and olfactory pathways were rare (Mulder *et al.*, 1994; Babic *et al.*, 1996). There were abundant sensory nerve endings distributed in subcutaneous. When PRV was injected in subcutaneous, it could infect CNS through sensory nerve (Brittle, Reynolds and Enquist, 2004).

As a model animal, mouse had been widely used for PRV research. Different infection routes resulted in various peculiar profiles. In many researches, infected mouse with inconsistent ways could show different pathological changes. In this study, in order to find the specific areas of virus distribution, we investigated the clinic signs, histopathological and immunohistochemical changes of mouse infected with PRV following intragastric administration, intranasal inoculation and subcutaneous inoculation.

MATERIALS AND METHODS

Virus: The recombinant virus PRV-Halv was presented by Professor Hanzhong Wang of China Academy of Sciences, Wuhan Institute of Virology. The titer of the virus was 2.5×10^7 TCID$_{50}$/mL.

Animal experiments: All experimental procedures involving animals were approved by the Laboratory Animal Monitoring Committee of Huazhong Agricultural University. Twenty four adult female Kunming mice in similar weight were divided into 6 groups randomly, housed under room temperature, 12 h alternating light-dark cycle, offered feed and water *ad libitum* for 2 days. Three groups were respectively injected through intragastric administration, intranasal and subcutaneous into the cervical skin with 0.1 mL of the stock virus at title 2.5×10^6 TCID$_{50}$/mL each mouse. Three groups were injected with the same volume phosphate buffer saline (PBS) through intragastric administration, intranasal and subcutaneous. All these 6 groups were fed in different cages. Clinical symptoms were observed and recorded 6 times every day. The moribund mice were humanely sacrificed and the tissues of heart, liver, spleen, lung, kidney, intestines, cerebrum, cerebellum and spinal cord were collected. Parts of the tissues were stored at -80°C for extracting DNA and other parts of tissues were fixed in 10% neutral-buffered formalin for 48 h.

PRV Detection by PCR: Tissues DNA were extracted by EasyPure Genomic DNA Kit (Transgen Biotech, China). The presence of PRV DNA in the tissue was assessed by PCR amplification of a 217-bp target sequence of *gE* gene. The sequence of the forward primer was 5-CGCCACCTGGGACTACACGC-3' and the reverse primer was 5'-GTGCTGGCG TTGGGCTCCTTC-3'. The reaction cocktail included Eastap 0.5 µL, Eastap buffer 2.5 µL, dNTP 1 µL, double distilled water 17 µL, forward primer 1 µL and reverse primer 1 µL. The reaction system was initially heated at 95°C for 10 min and denatured at 95°C for 30 sec, annealed at 58°C for 30 sec. the samples were then subjected to 32 cycles of amplification and maintained at 72°C for 10 min. PCR products were analysed by electrophoresis in 1% agarose gels.

Histopathology: The tissues were fixed in 10% neutral buffered formalin, dehydrated in a series of alcohols, and embedded in paraffin wax. Sections (4 µm) were prepared from paraffin wax-embedded tissues and stained with haematoxylin and eosin (HE). The sections were examined using the microscope (Leica DM300).

Immunohistochemical examination: Paraffin sections were placed in 3% H_2O_2 for 30 minutes to quench endogenous peroxidases. Antigen retrieval was accomplished by incubating the sections in citrate buffer for 30 minutes at 96°C. After washing, the sections were blocked for 1 h in 5% BSA and incubated with swine anti-PRV primary antibody overnight at 4°C (1:300, provided by Veterinary Pathology Lab, Huazhong Agricultural University, China). After washing, the sections were incubated with secondary antibody (HRP conjugated rabbit anti-swine 1:1000, Sigma) for 1 h. Finally, the sections were treated with chromogen (0.05% 3,3-diaminobenzidine tetrahydrochloride and H_2O_2) and counterstained with haematoxylin. The sections were examined by using microscope (Leica DM300).

RESULTS

Clinical signs and gross pathology: At 80 h post injection, the intragastric administration group started to show exciting and roughening of the fur. Thereafter, frequent scratching mainly at the skin on the head and face were observed. It resulted in skin ulceration and hypodermis exposure. Once clinical signs appeared, all the mice died within 12 h. Pruritus in intranasal group appeared at 72 h post injection (Fig. 1A), and pruritus in subcutaneous group appeared at 96 h post injection. In intragastric administration group, gross lesions included slightly hyperemia in lung and spleen. Hydrocephalus, the gyri were flattened and the sulci obliterated. Haemorrhagic and thinning were found in small intestine. In intranasal group, lung hemorrhage and hydrocephalus were observed. Hydrocephalus was the most common symptom in subcutaneous group. No gross lesions were observed in the mice of control group.

PCR for PRV detection: The results of the virus detection showed in Table 1. After intragastric administration infection, PRV was detected in spinal cord, cerebellum, cerebrum, lung and intestine. In intranasal group, PRV was detected in spinal cord, cerebrum, cerebellum, spleen, lung and kidney. And in the group of the subcutaneous, PRV was just detected in the spinal cord, cerebrum, kidney and cerebellum. All the control mice were negative.

Table 1: The distribution of PRV in mice after gavage, intranasal and subcutaneous infection

|	Intragastric administration		Intranasal		Subcutaneous	
	PCR	IHC	PCR	IHC	PCR	IHC
Heart	0/3	2/3	1/3	1/3	0/3	0/3
Liver	0/3	0/3	0/3	0/3	0/3	0/3
Spleen	0/3	0/3	2/3	2/3	0/3	0/3
Lung	1/3	0/3	1/3	2/3	0/3	0/3
Kidney	0/3	0/3	1/3	0/3	1/3	1/3
Intestinal	1/3	3/3	0/3	0/3	0/3	0/3
Cerebrum	1/3	1/3	3/3	0/3	2/3	2/3
Cerebellum	2/3	2/3	2/3	2/3	1/3	1/3
Spinal cord	3/3	3/3	3/3	3/3	3/3	3/3

Note: In this article, we said "Cerebrum" included of medulla oblongata and pons.

Histopathology: In the intragastric administration group, mild-to-severe pulmonary edema, hemorrhage and broadening interstitial filled with lymphocytes in lung were observed. While in the intestine, hemorrhages were observed and the intestinal villi showed mild rupturing (Fig. 1C). Mild hemorrhages were observed in the myocardium. Nonsuppurative encephalitis, characterized by multifocal areas of necrosis, pronounced gliosis, and neuronophagia were observed in cerebrum (Fig. 1E) and spinal cord. In intranasal group, mild hemorrhage was found in lung, brain and myocardium. Mild-to-severe interstitial pneumonia and nonsuppurative encephalitis mainly performed with neuronophagia were observed in brain (Fig. 1B, 1D). In subcutaneous group, nonsuppurative encephalitis consisted of glial proliferation, nerve cells atrophy and neuronophagia were observed in cerebrum (Fig. 1F), cerebellum and spind cord, no histopathological aberrations were detected in the other organs or tissue samples. No microscopic lesions were observed in the control groups.
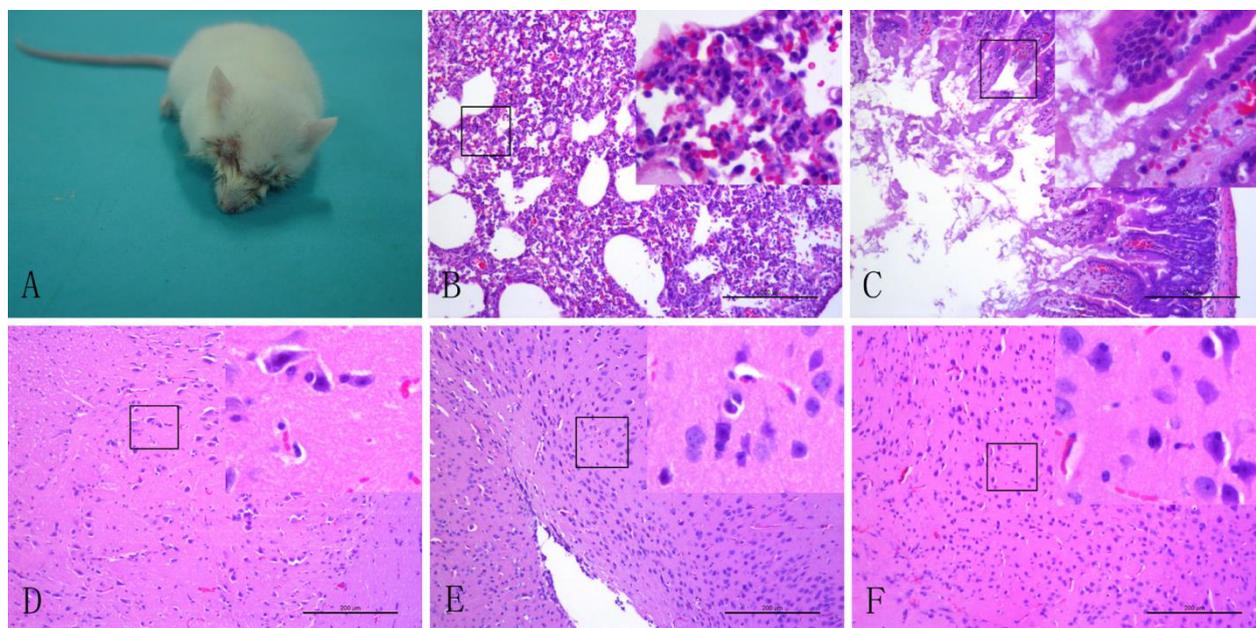
Fig. 1: The pathology change of mice in different injected routes; (**A**. The mouse of intranasal group frequent scratching mainly at the region of head and face were resulting in the skin breaking; **B**. Pulmonary edema, hemorrhage and broadening interstitial lung in intranasal group, HE; **C**. Mild rupturing of intestinal villi in intragastric administration group, HE; **D**. Neuronophagia of spinal cord in intranasal group, HE; **E**. Necrosis, pronounced gliosis and neuronophagia of cerebellum in intragastric administration group, HE; **F**. Pronounced gliosis, and neuronophagia were observed in cerebrum in subcutaneous group, HE. 400X)
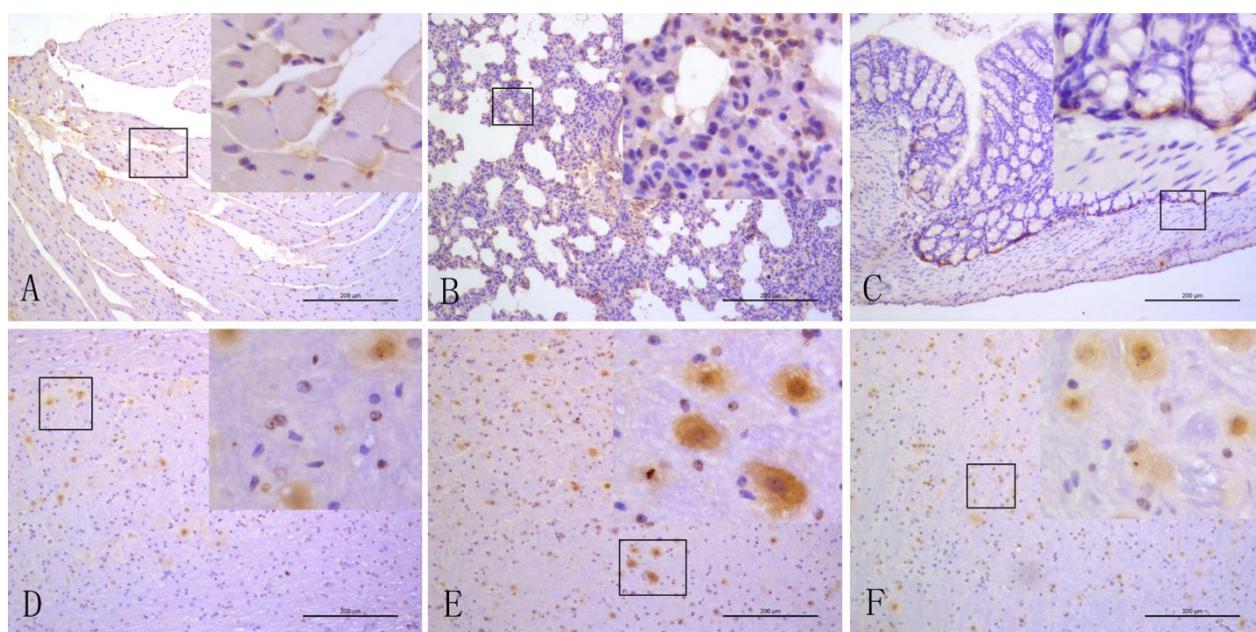
Fig. 2: The distribution of PRV antigens in different injected routes; (**A**. PRV antigens detected in heart muscle through intranasal injected, Immunochemistry (IHC); **B**. In intranasal group, pulmonary epithelial cells expressed PRV positive, IHC; **C**. PRV antigen detected in intestinal through intragastric administration injected, IHC; **D**. Virus antigen was detected in spinal cord through intranasal injected, IHC; **E**. Cerebellum expressed positive by intragastric administration injected, IHC; **F**. The positive signs were observed in cerebrum after subcutaneous injection, IHC, HE. 400X).

Immunochemistry: PRV infection of the mice was confirmed by using immunochemical staining (Table 1). In intragastric administration group, the appearance of viral antigens was located in a few neuronal cells and gliocytes in the brain hippocampus, most in the medulla oblongata and gray substance spinal cord (Fig. 2E). In the nervous system, the antigens were located in both nucleus and cytoplasm of the viral-antigen positive cells. PRV antigen was also detected in the intestinal submucosa (Fig. 2C), a few nucleuses of endothelial and myocardial cells. In intranasal group, PRV antigens were detected in the cytoplasm and nucleus of neuronal cells and gliocytes in the gray substance spinal cord and pons (Fig. 2D). And the PRV antigens were detected in the nucleuses of endothelial and myocardial cells (Fig. 2A), pulmonary epithelial cells (Fig. 2B), and lymphocytes in red pulp of the spleen. In subcutaneous group, the viral antigens were located in the sensory and motor cortex, prefrontal cortex (Fig. 2F), gray substance spinal cord and purkinje cells in the cerebellum. The antigens were distributed in both nucleus and cytoplasm of the viral-antigen positive cells. Lymphocytes in the splenic red pulp were positive for PRV-antigen treated group while no viral antigen was detected in the control groups.

DISCUSSION

PRV is a kind of highly neurotropism alphaherpesviruses. In clinic, PRV is initially replicated in the epithelial cells of the site viral infection. After local replication in the epithelial cells, these viruses gain access to the connective tissues and find their way to blood vessels and nerve endings (Glorieux et al., 2011). After access to the nerve endings, the encapsidated viral genome then spread directionally inside axons via microtubules to the cell bodies of sensory and autonomic ganglia. However, in nonnatural hosts, such as mouse, infection continues to spread to second-order and third-order neurons in the central nervous system (Olsen et al., 2006; Lyman et al., 2008; Kratchmarov et al., 2013). So, the distribution of PRV and the survival time of the mouse are dependent on the virulence and dose of the virus (Van Oirschot and Gielkens, 1984; Damann et al., 2006; Ye et al., 2014). PRV could infect haslet via splanchnic nerve from CNS (Brittle et al., 2004) and also via leukocytes and blood circulation (Lee et al., 2011).

When mouse was infected with PRV, it initially showed exciting, then slight bradykinesia and roughening of the fur. Thereafter, frequent scratching mainly at the virus-inject skin and hyperkinesias are observed, once clinical signs appear, the mouse would die within 24 h. In recent reports, the reason for mad itch is infection-induced, synchronous, and cyclical firing in PNS ganglia that project to the infected tissues, which results from fusion pore formation in unmyelinated axons of infected neurons (Yang et al., 1999; Granstedt et al., 2013). In this study, clinical signs appeared in the intranasal group at 72 h post injected and the mice died within 12 h. The clinical signs in intragastric administration group were appeared at 80 h post inoculation. At 96 h post inoculation, clinical signs appeared in subcutaneous group. It might mean that the appearance time of clinical sign depends on the length of axon transmission.

PRV would transport transneuronally via vagal and spinal viscerosensory neurons to postsynaptic target cells in the medullary dorsal vagal complex and thoracic dorsal horn (Ye et al., 2014), respectively, with subsequent transport to discrete regions of the medullary and pontine reticular formation, cerebellum, parabrachial nucleus, periaqueductal gray, thalamus, hypothalamus, amygdala, bed nucleus of the stria terminalis, and other central sites (Banihashemi and Rinaman, 2010; Li et al., 2015b). However, transport transneuronally was changed with the PRV strain, the strain of H129 did not transport transneuronally in the retrograde direction from infected neurons to central sources of presynaptic input (Rinaman and Schwartz, 2004). In general, virulence strain can propagate by transneuronal transfer in both the retrograde and anterograde directions, whereas the attenuated strain of PRV propagates only by retrograde (Ugolini, 2010). In the intragastric administration group, PRV was transported transneuronally via vagal and spinal viscerosensory neurons. At last, PRV was detected in intermediolateral cell column of thoracic, medulla oblongata and hippocampus. Via peristalsis, PRV was detected in intestine.

After intranasal inoculation with PRV, it propagates to first and second-order neurons in trigeminal, sympathetic, olfactory system and parasympathetic pathways. But in mice, olfactory system pathway is only rarely infected (Babic et al., 1996; Lee et al., 2012). In this experiment, PRV antigen was found in cerebellum and spinal cord by Immunochemistry. At the same time, PRV was found in cerebellum, cerebrum and spinal cord by PCR. PCR showed high sensitivity and specificity for detecting PRV. Therefore, small amounts of virus could be detected by PCR but not in IHC. The impact of the multiplicity of infection and the axonal milieu on the establishment of neuronal infection initiated from axons (Li et al., 2015a). And the distribution of PRV in the brain is virulence dependent and dose-dependent (Damann et al., 2006; Ugolini, 2010). Large dose injection and virulent PRV would result in mice death before amounts of viral particles reaching the CNS. Small dose injection and attenuated PRV would move to neuronal nuclei and keep silence in the first-order neurons (Olsen et al., 2006; Ye et al., 2014). In this study, PRV antigen was not detected in cerebrum, which might be caused by large dose injection or virulent. Via leukocyte and blood circulation, PRV was distributed in heart, spleen, lung and kidney. PRV could also infect these organs by anterograde spread along sympathetic neurons from thoracic spinal cord, by retrograde spread from the general visceral afferent pathways that connect the lumbar spinal cord and kidneys (Brittle et al., 2004).

After subcutaneous inoculation with PRV, it invades sensory neurons innervating the skin (dorsal root ganglia), and transported to the dorsal horn of spinal cord. Motor neurons originating in the ventral horn of the spinal cord projecting to the skin are also infected. Then PRV retrograde spreads from spinal cord to the raphe magnus nucleus of the brainstem. At last, the periaqueductal gray of the midbrain and the movement of the forebrain cortex are also infected. And the virus injected in subcutaneous of neck skin could spread through cranial never in CNS (Brittle et al., 2004; Koyuncu et al., 2015). In the study,

PRV was detected in spinal cord and prefrontal cortex, which might indicate that PRV can result in neuroinvasion via sensory, motor and cranial nerve.

Conclusions: In conclusion, after intragastric infection, PRV mainly distributed in intermediolateral cell column of thoracic, medulla oblongata hippocampus, intestinal submucosa and myocardial cells. Through intranasal infection, PRV was detected in gray substance spinal cord pons, myocardial cells pulmonary epithelial cells and spleen. And PRV can transfer to the sensory and motor cortex, prefrontal cortex, gray substance spinal cord, purkinje cells in the cerebellum and the spleen after subcutaneous injection.

Authors contribution: In this article, Zuquan Fu and Wanpo Zhang conceived and designed the project/study. Yanchaun Li and Xiaoli Liu executed the experiment. Xueying Hu, Changqin Gu, Guofu Cheng analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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