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Competitive replication kinetics and pathogenicity in pigs co-infected with historical and newly invading classical swine fever viruses



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ABSTRACT

Classical swine fever (CSF), an economically important and highly contagious disease of pigs, is caused by classical swine fever virus (CSFV). In Taiwan, CSFVs from field outbreaks belong to two distinct genotypes. The historical genotype 3.4 dominated from the 1920s to 1996, and since 1996, the newly invading genotype 2.1 has dominated. To explain the phenomenon of this virus shift in the field, representative viruses belonging to genotypes 2.1 and 3.4 were either inoculated alone (single infection) or co-inoculated (co-infection), both *in vivo* and *in vitro*, to compare the virus replication and pathogenesis. In pigs co-infected with the genotype 2.1 TD/96/TWN strain and the genotype 3.4 94.4/IL/94/TWN strain, the newly invading genotype 2.1 was detected earlier in the blood, oral fluid, and feces, and the viral loads were consistently and significantly higher than that of the historical genotype 3.4. In cell cultures, the ratio of secreted virus to cell-associated virus of the genotype 2.1 strain was higher than that of the genotype 3.4 strain. This study is the first to demonstrate a possible explanation of virus shift in the field, wherein the newly invading genotype 2.1 replicates more efficiently than did genotype 3.4 and outcompetes the replication and pathogenicity of genotype 3.4 in pigs in the field.

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1. Introduction

Classical swine fever (CSF) is a highly contagious disease of swine caused by classical swine fever virus (CSFV), an enveloped RNA virus belonging to the genus *Pestivirus* of the family *Flaviviridae* (Simmonds et al., 2011). The CSFV genome consists of a single, positive-stranded RNA of approximately 12.3 kb encoding for a polyprotein of 3898 amino acids, which is flanked by 5' and 3' non-translated regions (NTR). The translated polyprotein is processed by viral as well as cellular proteases to the mature viral proteins of four structural (C, E^{rns}, E1, and E2) and eight nonstructural proteins (NP^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Lindenbach et al., 2013).

The CSFVs are classified into highly virulent, moderately virulent, low virulent, and avirulent strains (van Oirschot, 1988). Highly virulent strains cause acute disease with high morbidity and mortality, irrespective of the age and breed of infected pigs, while moderately virulent strains generally cause subacute or chronic disease in infected pigs, in which host factors play a role in the outcome of infection. Low virulent strains cause mild disease or subclinical infection (van Oirschot, 1999). Avirulent strains, such as the vaccine strains, are completely non-pathogenic but induce a protective immunity (Huang et al., 2014). The molecular determinants of CSFV virulence have been defined by reverse genetics technology (Leifer et al., 2013). It appears that seven proteins of NP^{pro}, C, E^{rns}, E1, E2, p7, and NS4B, influence CSFV replication efficiency or pathogenicity in pigs (Ji et al., 2015). Analysis of the replication kinetics of CSFVs has shown no significant differences between the strains regarding RNA replication or protein synthesis, but the ratio of secreted virus to cell-associated virus is higher for highly virulent strains (Mittelholzer et al., 2000).

Genotyping has been useful for tracing the spread of CSFVs (Greiser-Wilke et al., 2000). The three regions of the CSFV genome most commonly analyzed for that purpose are 150 nucleotides of the 5'-NTR, 190 nucleotides of the E2 envelope glycoprotein gene,

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and 409 nucleotides of the NS5B polymerase gene (Paton et al., 2000). CSFVs are divided into three groups with three or four genotypes in each group: 1.1, 1.2, 1.3, 1.4; 2.1, 2.2, 2.3; 3.1, 3.2, 3.3, 3.4 (Paton et al., 2000; Postel et al., 2013). Group 1 contains most of the historical strains, including vaccine strains, distributed in most regions of the world. Group 2 contains most of the currently prevalent strains, whose infections have increased and caused epidemic infection since the 1980s. Group 3 contains most of the strains distributed in separated geographic regions (Beer et al., 2015; Paton et al., 2000; Sakoda et al., 1999).

In Taiwan, CSFVs from field outbreaks belong to two distinct genotypes: historical strains of genotype 3.4, such as strain 94.4/IL/94/TWN, first present prior to 1920s and dominating until 1996; and newly invading strains of genotype 2.1, such as strain TD/96/TWN, first present in 1994 and dominating since 1996 (Deng et al., 2005; Lin et al., 2007). This shift in CSFV populations from genotypes 3 or 1 to genotype 2 has been similarly observed in Europe and other areas of Asia in recent years (Cha et al., 2007; Greiser-Wilke et al., 2000; Shivaraj et al., 2015; Tu et al., 2001). The reasons for this shift, however, remain unclear.

To further the understanding on the characteristic of diverse CSFV genotypes and to successfully eradicate CSFV, it is important to explain the phenomenon of the virus shift in the field. In the present study, viruses belonging to genotypes 2.1 and 3.4 were analyzed *in vitro* and *in vivo* to compare their abilities in virus replication and pathogenesis.

2. Materials and methods

2.1. Cells and viruses

Porcine kidney-15 (PK-15) cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in 5% CO₂. Two representative CSFV strains belonging to genotypes 2.1 and 3.4, propagated in the PK-15 cells, were used in this study: TD/96/TWN (genotype 2.1) and 94.4/IL/94/TWN (genotype 3.4) (Deng et al., 2005; Lin et al., 2007; Paton et al., 2000).

2.2. Monoclonal antibodies (mAbs) specific for CSFV E2

Two mAbs, T6 and L71, produced by the Animal Health Research Institute, Taiwan, were used in this study. The mAb T6 recognizes the CSFV genotype 2.1 TD/96/TWN strain, but not the genotype 3.4 94.4/IL/94/TWN strain. The mAb L71 recognizes the 94.4/IL/94/TWN strain, but not the TD/96/TWN strain.

2.3. Animal infections

Twelve 8-week-old specific pathogen-free (SPF) pigs were randomly separated into three groups of four pigs. Pigs were inoculated as follows: with 1 ml of the TD/96/TWN strain alone (single infection), with 1 ml of the 94.4/IL/94/TWN strain alone (single infection); and with 1 ml of TD/96/TWN and 1 ml of 94.4/IL/94/TWN simultaneously (co-infection). All inoculated viruses were adjusted to 10⁶ TCID₅₀ (tissue culture infectious dose 50%) per ml. Pigs were inoculated intramuscularly to ensure that two strains could enter hosts simultaneously. Pigs were housed in completely separated negative air-pressure isolation units. For animal welfare reasons, pigs were euthanized when they were moribund and unable to stand up. All remaining pigs were euthanized at 13 days post infection (dpi), the end of the experimental period.

This animal experiment was approved by the Institutional Animal Care and Use Committee of the Animal Health Research Institute (A02040).

2.4. Clinical signs, body temperature, and sampling procedures

Rectal temperature was recorded daily through the experimental period to day 13. Fever was defined as temperature higher than 40 °C. For evaluation of clinical signs, the ten parameters described by Mittelholzer et al. (2000) were scored from 0 to 3 to represent normal to severe CSF symptoms. The scores for each pig were summed into a total score for each day. Blood, oral swabs and fecal swabs were collected prior to inoculation and then at a 2-day interval post infection. Swabs were weighed before and after sampling to normalize the viral loads. Each swab was immersed in 2 ml of phosphate buffered saline (PBS) and centrifuged at 3000 × g for 10 min. After the centrifugation, the supernatant obtained was stored at −70 °C. Necropsies were performed after euthanasia or death, and tissue samples of tonsil, submandibular and mesenteric lymph nodes, heart muscle, lung, liver, spleen, kidney, bladder, and cerebrum were collected from all animals.

2.5. Leukocyte count

Leukocyte counts were performed using the Medonic CA® 620 coulter counter (Boule Medical AB, Stockholm, Sweden). Leukopenia was defined as an amount of leukocyte lower than 11 × 10⁹ cells/l blood.

2.6. Quantitative reverse transcription multiplex real-time polymerase chain reaction (RT-MRT-PCR)

RNAs were extracted using the QIAamp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany). The specific genotyping RT-MRT-PCR was performed to detect and genotype CSFV as described by Huang et al. (2009), who demonstrated no inter-genotypic cross-reactivity among different CSFV strains. The viral loads, determined by the RT-MRT-PCR, were expressed as log viral genome copies/μl.

2.7. Multistep growth curve

PK-15 cells seeded in 25T flasks were infected at a multiplicity of infection (MOI) of 0.1 with the TD/96/TWN strain alone (single infection), with the 94.4/IL/94/TWN strain alone (single infection), or with both TD/96/TWN and 94.4/IL/94/TWN simultaneously (co-infection). After incubation for 1 h at 37 °C, unbound viruses were removed and the cells were washed three times with PBS before being incubated in fresh medium at 37 °C to synchronize the infection. Viruses were harvested at 0, 3, 6, 12, 24, 48, and 72 h post infection (hpi). For titrating secreted virus, the supernatant was harvested, centrifuged at 3000 × g for 10 min, and the debris discarded. For cell-associated virus, infected cells were suspended in a volume of PBS corresponding to the removed supernatant and freeze-thawed twice, after which the lysate was clarified by centrifugation at 3000 × g for 10 min before titration.

2.8. Viral attachment

PK-15 cells seeded in 12-well plates were stored for 2 h at 4 °C before being infected at an MOI of 10 with the TD/96/TWN strain alone, with the 94.4/IL/94/TWN strain alone, or with both TD/96/TWN and 94.4/IL/94/TWN simultaneously. After incubation for 1 h at 4 °C, unbound viruses were removed and the cells were washed three times with PBS. Fresh PBS was added and the cells were stored at −80 °C for more than 2 h. The cells were thawed at 4 °C, and the supernatant was clarified by centrifugation at 3000 g for 10 min to remove debris.

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