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Development of a Chimeric Strain of Porcine Reproductive and Respiratory Syndrome Virus with an Infectious Clone and a Korean Dominant Field Strain

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The K418 chimeric virus of porcine reproductive and respiratory syndrome virus (PRRSV) was engineered by replacing the genomic region containing structure protein genes of an infectious clone of PRRSV, FL12, with the same region obtained from a Korean dominant field strain, LMY. The K418 reached 10^6 $TCID_{50}$/ml of viral titer with similar growth kinetics to those of parental strains and had a cross-reactive neutralizing antibody response to field serum from the entire country. The chimeric clone pK418 can be used as a practical tool for further studying the molecular characteristics of PRRSV proteins through genetic manipulation. Furthermore, successful construction of the K418 will allow for the development of customized vaccine candidates against PRRSV, which has evolved rapidly in Korea.

Keywords: PRRSV, reverse genetics, infectious clone, chimera, customized vaccine

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), which causes a reproductive and respiratory disease in pigs, contains a positive-sense single-strand RNA genome measuring approximately 15.4 kb. The virus is classified into two genotypes, a North American (NA) type and a European (EU) type. The genome of PRRSV contains nine open reading frames (ORFs). ORF1a and ORF1b occupy approximately 80% of the viral genome and encode nonstructural proteins (NSPs) (Meulenberg *et al.*, 1993). The remaining ORFs encode glycosylated structure proteins, including the glycoprotein (GP)2, GP3, GP4, and GP5, unglycosylated membrane (M) protein and nucleocapsid (N) protein (Meulenberg, 2000). Previous studies indicated that B-cell epitopes for viral neutralization appeared to reside on structure proteins, including GP3, GP4, GP5, and M (van Nieuwstadt *et al.*, 1996; Yang *et al.*, 2000; Cancel-Tirado *et al.*, 2004; Ansari *et al.*, 2006; Plagemann, 2006).

PRRS is characterized by reproductive failure, including aborted, stillborn, mummified and weak-born piglets, and respiratory disorder, which can result in high death rates in suckling and weaned pigs. PRRS is responsible for significant economic losses for the swine industry worldwide (Neumann *et al.*, 2005). Control methods including management of incoming replacement gilts, implementation of biosecurity protocols and vaccination have been applied to reduce the risk of PRRSV outbreak. Among them, vaccination has been considered as the most effective tool for preventing the disease (Thanawongnuwech and Suradhat, 2010). However, current commercial PRRSV vaccines have the drawback of featuring a high level of antigenic variation between the vaccine and field strains of PRRSV (Meng, 2000). Heterologous strains of PRRSV compared to a vaccine strain possessing antigenic diversity resulting from amino acid sequence variation in GP5 have been constantly isolated and reported from Korea (Cha *et al.*, 2006; Yoon *et al.*, 2008; Kim *et al.*, 2012; Choi *et al.*, 2013). Generally, field strains, which are the most frequently isolated from regional areas, have been used to develop vaccines in each country that has had an endemic PRRSV outbreak.

Reverse genetics (RG) technology is a practical system to study virus characteristics, *in vivo* pathogenesis and viral protein function. The manipulation of viral RNA genomes is generally difficult due to the instability of RNA genomes and the lack of research tools for direct RNA editing. However, with RG technology, the modification of infectious RNA viruses can be easily achieved. In previous studies, RG technology has been applied to the genetic modification of genomes of positive-sense and negative-sense RNA viruses and to rescue mutant viruses from cDNA infectious clones (Taniguchi *et al.*, 1978; Racaniello and Baltimore, 1981; Castrucci and Kawaoka, 1995).

RG technology has widespread implications in the fields of virology and vaccinology (Ito *et al.*, 2001; Collins and Murphy, 2005; Almazan *et al.*, 2013). Using RG technology, a highly pathogenic strain of a virus can be attenuated by the construction of a chimeric virus with highly and weakly pathogenic viruses. In addition, a chimera virus can serve as a viral vector expressing heterologous antigen. The infectious clone of PRRSV has been developed and used in previous studies (Nielsen *et al.*, 2003; Truong *et al.*, 2004; Yoo *et al.*, 2004; Lee *et al.*, 2005; Fang *et al.*, 2006). In previous studies, diverse chimera viruses of PRRSV have been generated using RG technology to manipulate viral genomes

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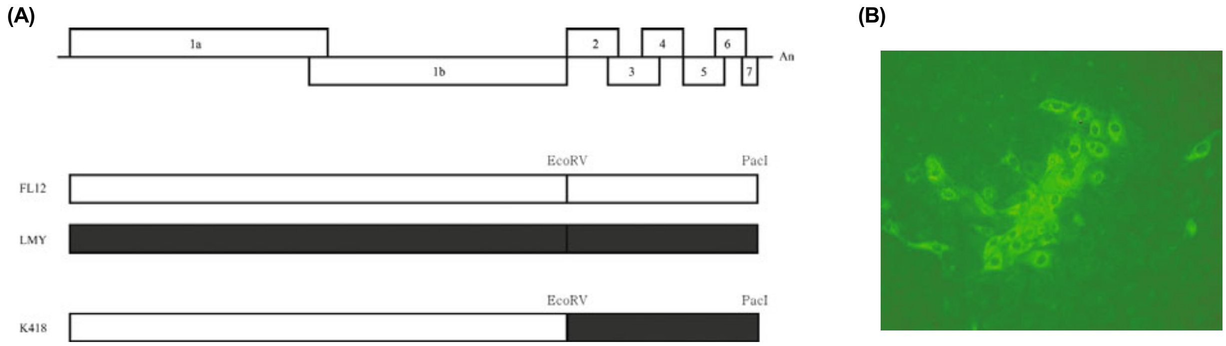

Fig. 1. A schematic genome figure of chimeric and parental strains and immunofluorescence assay result of K418. (A) The general genomic arrangement of PRRSV is shown at the top of the figure. The genomic regions of parental viruses, FL12 (blank) and LMY (gray), and the locations of the restriction enzyme sites used for genetic manipulation are indicated. (B) Immunofluorescence assay (IFA) using the N protein of PRRSV-specific monoclonal antibody was performed to detect viral replication after the transfection of MARC-145 cells.

to determine the viral protein involved in the pathogenicity of field strains in pigs (Kwon *et al.*, 2006; Zhou *et al.*, 2012; Ni *et al.*, 2013). However, RG technology has been used mainly to characterize the function of viral proteins but has not been used to develop recombinant vaccine candidates against PRRSV. In this study, a chimeric virus was constructed using an infectious clone of PRRSV containing genomes of the FL12 strain and a Korean dominant field strain, LMY. The chimeric virus, named K418, contains NSPs from the FL12 strain and structure proteins from the LMY strain.

Materials and Methods

Cells and virus

MARC-145 cells were used to rescue virus and to determine viral growth kinetics. The PRRSV field isolate, LMY, was isolated at the Animal, Plant and Fisheries Quarantine and Inspection Agency (Korea) from a case of PRRSV infection associated with clinical disease. Virus titers were calculated and expressed as tissue culture infectious doses 50 ($TCID_{50}$)/ml.

Construction of a chimeric clone and recovery of chimeric virus

The PRRSV infectious cDNA clone, pFL12, was generously provided by Dr. Fernando Osorio and Dr. Asit Pattnaik of the University of Nebraska-Lincoln (Truong *et al.*, 2004). A genomic region including whole-structure genes from the LMY strain of PRRSV was amplified using reverse transcription-polymerase chain reaction (RT-PCR) with a primer pair of 5′-GTGGATGCTTTCACGGAGTTC-3′ and 5′-CACACTTAATTAACGTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTAATTTCGGCCGTGT GGTTC-3′. The genomic region containing the majority of ORF2 and all of the other structure protein genes of the pFL12 plasmid was replaced with the amplified structure protein genes of the LMY strain to generate a chimeric clone pK418 using restriction enzymes *Eco*RV and *Pac*I (NEB, USA) (Fig. 1A). Virus recovery was performed as previously described with a slight modification (Truong *et al.*, 2004). MARC-145 cells were resuspended in phosphate buffered saline (PBS) and transfected by Gene Pulser Xcell (Bio-Rad, USA) at 250 V and 975 uF in a 4.0-mm cuvette with generated RNA transcripts and placed in a six-well plate with culture media. Two days after transfection of MARC-145 cells with *in vitro* transcribed RNA, cells were examined using immunofluorescence assay (IFA) to detect the expression of the N protein of PRRSV.

Viral growth kinetics

Growth kinetics of the chimeric virus and parental strains were performed by infection of MARC-145 cells seeded in a 6 well plate. Culture supernatant was collected at 12, 24, 48, 72, and 96 h post-infection, and viral titers in the supernatant were determined and expressed as $TCID_{50}$/ml in MARC-145 cells.

Serum neutralization assay

To investigate whether the LMY strain is a dominant strain of the NA genotype of PRRSV in Korea, serum neutralization assays were performed using 170 field serum samples collected from various regional areas. The serum neutralization assay was performed as previously described (Truong *et al.*, 2004). The serum neutralization titers were determined using IFA and were expressed as the reciprocal of the serum dilution that produced a 50% or higher reduction in the wells.

Results

Generation of K418 chimeric virus

The chimeric full-length cDNA clone, pK418, was transfected into MARC-145 cells. At 48 h posttransfection, the infected cells were analyzed by IFA using antibody against N protein of PRRSV. The chimeric virus, K418 expressed viral protein (Fig. 1B) and gradually replicated into the neighboring cells (data not shown). Recent studies have indicated that MARC-145 cells (African green monkey kidney), which are a permissive cell line for PRRSV, could be electroporated with

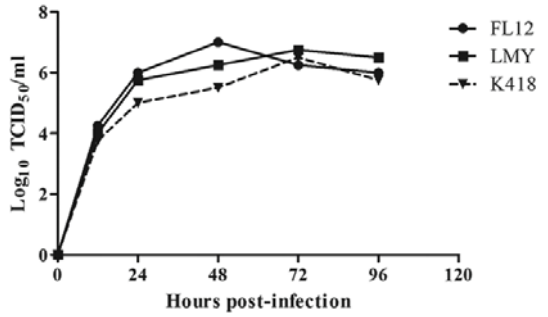

Fig. 2. Growth kinetics of chimeric and parental strains. The chimeric virus, K418 (inverted triangle), and parental viruses, FL12 (circle) and LMY (square), were used to analyze their growth kinetics in MARC-145 cells.

RNA for the recovery of infectious PRRSV as efficiently as baby hamster kidney (BHK)-21 cells (Truong *et al.*, 2004; Ansari *et al.*, 2006). These results showed that the chimeric virus, K418, recovered and replicated well in MARC-145 cells.

Growth properties of the chimeric virus

Upon the initial development of CPE, the supernatant from the electroporated cells was collected and infected into MARC-145 cells to propagate recovered viruses. The once-passaged culture supernatant produced 80% CPE at 5 days post-infection and yielded virus titers of 10^6 $TCID_{50}$/ml. The multi-step growth kinetics of the chimera virus was compared to those of parental strains in MARC-145 cells (Fig. 2). Genetic engineering did not greatly affect viral replication. The result of the growth kinetics study demonstrated that the final titers of the chimera and parental viruses were similar, whereas the chimeric virus showed slightly delayed viral replication.

Cross neutralization of field samples by K418 chimeric virus

The serum samples were divided into three groups depending on the K418-specific neutralizing antibody titers, cross-reactive, partially cross-reactive and resistant. The neutralizing antibody titers of cross-reactive group were greater than 1:8, the titers of partially cross-reactive group were 1:8 and the titers of resistant group were less than 1:8. The cross-reactive samples against K418 virus, including partially cross-reactive ones, accounted for 27.06% whereas cross-reactive samples against MLV which widely used commercial live vaccine accounted for 5.88% of the total field samples (Table 1).

Table 1. The neutralizing antibody response of field serum samples against the chimeric virus, K418 and MLV

Cross-reactivity	SN titer	Number of serum (%)	
		Against K418	Against MLV
Cross-reactive	>1:8	18 (10.59%)	3 (1.76%)
Partially cross-reactive	1:8	28 (16.47%)	7 (4.12%)
Resistant	<1:8	124 (72.94%)	160 (94.12%)
Total		170	

Discussion

RG technology has been used to produce numerous vaccine candidates against other viruses, including the influenza, human respiratory syncytial, Newcastle disease and foot-and-mouth disease viruses (Castrucci and Kawaoka, 1995; Collins and Murphy, 2005; Hu *et al.*, 2009; Blignaut *et al.*, 2011). The chimera viruses developed using RG technology could be used as a multivalent vaccine to control diseases caused by rapidly changing RNA viruses. In this study, a chimeric virus was constructed using an infectious clone of PRRSV, FL12, and a Korean dominant field strain, LMY, using RG technology. Many chimeric or mutant viruses of PRRSV have shown reduced viral titers relative to those of parental viruses (Bentley *et al.*, 2013; Ni *et al.*, 2013). However, K418 produced a viral yield similar to the yields of parental viruses. These results might be caused by the different characteristics of the strains used in this study, FL12 and LMY. The two strains have previously been incorporated into MARC-145 cells through multiple passages. Therefore, the recovered chimeric virus, K418, may have inherited its susceptibility to growth conditions in MARC-145 cells from parental viruses.

K418 showed a cross-reactive neutralizing antibody response against a considerable proportion of field serum samples collected from the entire country. Generally, protection against PRRSV in pigs can be established starting from the serum neutralization titers of 1:8 (Osorio *et al.*, 2002). Korean field strains of PRRSV were classified into NA or EU genotype with similar rate. There is a considerable antigenic variability between the NA and EU strains of PRRSV (Cha *et al.*, 2006; Yoon *et al.*, 2008; Kim *et al.*, 2012; Choi *et al.*, 2013). Furthermore, sequence diversity on the ORF5 decreased serological cross-reactivity between PRRSV strains belonging to the same genotype (Kim *et al.*, 2013). The MLV containing a representative attenuated strain of NA type virus is the most commonly used PRRSV vaccine worldwide in field. The MLV virus was frequently isolated from vaccinated pigs in fields. However, only 5.88% of the total PRRSV ELISA positive serum samples neutralized MLV, while 27.06% of PRRSV ELISA positive serum samples neutralized the chimeric virus, K418. The serological cross-reactivity against the K418 virus suggests that the humoral immune response induced by the chimeric virus would cross-react with approximately half percentage of the NA field strain of PRRSV in Korea. The polyclonal antiserum against the K418 neutralized the LMY strain, but not the FL12 and MLV strain of PRRSV (Lee *et al.*, manuscript in preparation). These results supported that the chimeric virus would be more effective as a potential regional vaccine candidate to protect pigs than the MLV vaccine.

To generate customized vaccine candidate, the genomic region of established PRRSV infectious clone encoding structure proteins that play a critical role in the virus neutralizing response were replaced with the same genomic region from a Korean dominant field strain of PRRSV. It is highly possible that K418 would induce a host immune response, which can broadly cross-protect most of the NA genotype of Korean field strains of PRRSV, because the virus expresses all structure proteins from one of the current dominant field

strains of PRRSV. Further studies are in progress to determine a possible application of K418 as a vaccine candidate in Korea and to evaluate the efficacy of other genetically modified K418 viruses.

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