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Adaptions of field PRRSVs in Marc-145 cells were determined by variations in the minor envelope proteins GP2a-GP3

Hong-Liang Zhang[a,1], Yan-Dong Tang[a,1], Chun-Xiao Liu[a], Li-Run Xiang[a], Wen-Li Zhang[a], Chao-Liang Leng[b], Qian Wang[a], Tong-Qing An[a], Jin-Mei Peng[a], Zhi-Jun Tian[a,*], Xue-Hui Cai[a,*]

[a] State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150001, China
[b] Henan Key Laboratory of Insect Biology in Funiu Mountain, Henan Provincial Engineering Laboratory of Insects Bio-reactor, China-UK-NYNU-RRes Joint Laboratory of Insect Biology, Nanyang Normal University, Nanyang 473061, China

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ABSTRACT

The recent rapid evolution of PRRSVs has resulted in certain biological characteristic changes, such as the fact that an increasing number of field PRRSVs can be isolated from PAMs but not from Marc-145 cells. In this study, we first isolated Marc-145-unadaptive field PRRSV strains from PAMs; sequence analysis showed that these PRRSVs belong to the HP-PRRSV (lineage 8) branch or NADC30-Like (lineage 1) branch. We further found major variations in ORF2-4 regions. To explore the viral adaptation mechanisms in detail, we constructed a full-length cDNA clone of MY-376, a Marc-145-unadaptive PRRSV. Construction of serially chimeric viruses of HuN4-F112 (a Marc-145-adaptive strain) and MY-376 demonstrated that variation in the minor envelope protein (GP2a and GP3) complex is a main determinant of PRRSV tropism for Marc-145 cells.

1. Introduction

Porcine reproductive and respiratory syndrome viruses (PRRSVs) are usually divided into two species: PRRSV-1 (type 1 genotype of European origin) and PRRSV-2 (type 2 genotype of North American origin). The PRRSV genome is approximately 15 kb in length, containing a 5′-untranslated region (5′-UTR), at least 10 open reading frames (ORF1a, ORF1b, ORF2a, ORF2b, ORF3-4, ORF5a and ORF5-7) and a 3′-UTR (Firth et al., 2011; Johnson et al., 2011). ORF1a and ORF1b, representing almost 75% of the viral genome, encode the polyproteins pp1a and pp1ab, which are cleaved into at least 14 non-structural proteins (Fang et al., 2012; Snijder and Meulenberg, 1998). ORF2a, ORF2b and ORF3-4, encoding the proteins GP2a, E, GP3 and GP4, respectively, are the minor structural proteins of PRRSV. ORF5, -6, and -7 encode the structural proteins GP5, M and N, respectively. Multimeric complexes of minor glycoproteins with or without the GP5 and M proteins are involved in direct interaction with the cell surface receptor CD163 (Das et al., 2010). In addition, PRRSV GP5/M complexes can interact with heparin, indicating their role in attachment of PRRSV to alveolar macrophages (Delputte et al., 2002). In addition, strong interaction between GP4 and GP5 has been reported (Das et al., 2010), and both GP2a and GP4 interact with all other viral glycoproteins, forming a multiprotein complex (Das et al., 2010).

PRRSV has a restricted cell and host tropism, and at least six putative PRRSV receptors have been described, including heparan sulfate (HS), vimentin, CD151, CD163 (scavenger receptor for the hemoglobin-haptoglobin complex), sialoadhesin (Sn, CD169), and DC-SIGN (CD209). Two of these receptors, Sn and CD163, are the most extensively studied; their ligands are the GP5/M heterodimer complex (Van Breedam et al., 2010) and a minor structural protein complex (Das et al., 2010; Tian et al., 2012), respectively. Porcine alveolar macrophages (PAMs) and blood monocytes are known to be susceptible to PRRSV infection. MA104, CL2621 and Marc-145 cells of the African green monkey are also permissive to PRRSV infection (Bautista et al., 1993; Benfield et al., 1992; Kim et al., 1993). Although Marc-145 cells are to date the cells most extensively used for PRRSV isolation, an increasing number of PRRSV strains cannot be isolated from Marc-145 cells.

The current PRRSV epidemiological situation in China is quite complex, with five PRRSV lineages existing in mainland China since 1996 (Gao et al., 2017). The rapid variation and recombination of PRRSV is accompanied by changes in its biological characteristics, including pathogenicity (Liu et al., 2017a, b; Sun et al., 2016; Wang et al., 2018; Zhao et al., 2015), immune escape (Bai et al., 2016; Bian et al., 2017; Zhang et al., 2016) and viral tropism in cell culture (Chen et al., 2015; Leng et al., 2014; Zhang et al., 2017). In the present study, we

* Corresponding authors.
E-mail addresses: tzj@hvri.ac.cn (Z.-J. Tian), cai139@hvri.ac.cn (X.-H. Cai).
[1] Both authors contributed equally to this work.

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show that an increasing number of PRRSV strains in China since 2014 fail to infect Marc-145 cells. Our results also indicate that the major variations in non-Marc-145-adaptive PRRSVs are located in ORF2-7 gene regions, especially in ORF2-4. Therefore, we hypothesize that these ORF2-4 variations are the major reason for the lack of Marc-145 cell infectivity of these PRRSVs.

2. Material and methods

2.1. Viruses, cells and antibody

HuN4-F112 is a live-attenuated vaccine strain derived from consecutive passage of Marc-145 cells. MY-376 (accession number KJ609517) is a wild-type strain that was isolated from PAMs in 2014. PAMs were obtained from 5-week-old specific pathogen-free (SPF) pigs (de Abin et al., 2009) and cultured in RPMI 1640 medium (Gibco BRL Co. Ltd., USA) supplemented with 10% fetal bovine serum (FBS; ExCell Bio., Australia). Marc-145 and BHK-21 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL Co. Ltd., USA) supplemented with 10% FBS. All cells were cultured in an incubator at 37 °C under 5% CO_2. A monoclonal antibody specific for the PRRSV M protein was prepared in our laboratory (Wang et al., 2014). An indirect immunofluorescence assay (IFA) was performed using fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Zsbio, Beijing, China) as the secondary antibody.

2.2. Phylogenetic analysis and sequence alignment

The sequence similarity of whole genomes, ORFs and deduced amino acids of viral proteins were assessed using the ClustalW method in Lasergene software (version 7.1) (DNASTAR Inc., Madison, WI, USA). Multiple sequence alignments were conducted using ClustalW in MEGA 6.0. Phylogenetic trees were also constructed in MEGA 6.0 using the neighbor-joining method with 1000 bootstraps. Simplot (version 3.5.1) was employed to test recombination within a 500-bp window, sliding along the genome alignments with a 20-bp step size.

2.3. Construction of the full-length MY-376 cDNA clone and chimeric clones

An infectious PRRSV strain clone was constructed as described previously (Zhang et al., 2011). The strategy used for constructing the full-length MY-376 strain cDNA clone is illustrated in Fig. 2A. The modified pBluescript II SK (+) construct, containing the SP6 polymerase promoter and two non-viral guanosine residues, was used as the vector (Zhang et al., 2011). Five fragments covering the complete MY-376 genome were amplified with Phusion® High-Fidelity DNA Polymerase (NEB, New England Biolabs) according to the manufacture's protocol. A synonymous mutation (8798 C→T) creating the restriction enzyme site ClaI was used as a genetic maker to differentiate the cloned and parental viruses. Six restriction enzymes (PacI, MfeI, NheI, ClaI, EcoRV and SwaI (NEB, New England Biolabs)) were utilized to construct the full-length MY-376 cDNA clone (designated pMY-376). The completely assembled cDNA clone was verified by sequencing at Comate Bioscience (Jilin, China).

The strategy applied for construction of the chimeric full-length cDNA clone is illustrated in Figs. 3A, Fig. 4A, Fig. 5A and Fig. 6A. Target fragments were prepared by PCR or overlapping extension PCR with Phusion® High-Fidelity DNA Polymerase, and chimeric plasmids were prepared by inserting the abovementioned target fragments between EcoRV and SwaI sites. The following chimeric plasmids with pHuN4-F112 and pMY-376 as the backbones were designed: rHuN4-F112-TORF2-7 and rMY-376-TORF2-7; rHuN4-F112-TORF2-4, rMY-376-TORF2-4, rHuN4-F112-TORF5-7, and rMY-376-TORF5-7; and rHuN4-F112-TORF2a, rMY-376-TORF2a, rHuN4-F112-TORF3, rMY-376-TORF3, rHuN4-F112-TORF4, rMY-376-TORF4; rHuN4-F112-TORF2a-ORF3 and rMY-376-TORF2a-ORF3. Chimeric cDNA clones were verified by sequencing at Comate Bioscience (Jilin, China).

2.4. Rescue and identification of parental and chimeric viruses

Parental and chimeric viruses were rescued as previously described (Zhang et al., 2011). First, in vitro transcription was performed by linearizing each full-length cDNA clone plasmid with the restriction enzyme SwaI (New England Biolabs) for more than 12 h, followed by purification with Cycle-Pure Kit (OMEGA). The linearized plasmid DNA was transcribed using the mMessage High Yield Capped RNA Transcription kit (Ambion, USA) according to the manufacturer's instructions. Second, the transcription product was transfected into BHK-21 cells using 12 μl of the DMRIE-C reagent (Invitrogen, U.S.) and 1 ml of opti-MEM (Gibco, USA) according to the manufacturer's protocol for 4 h and then washed twice with 2% DMEM; 2 ml of 2% DMEM was then added for 24 h. Finally, the supernatant harvested at 24 h post-transfection was serially passaged in PAMs or Marc-145 cells for infection. The rescued viruses were confirmed by PCR, cytopathic effects (CPEs) and IFA using the anti-M monoclonal antibody 3F7.

2.5. Growth kinetics evaluation

The viral growth properties of rescued viruses were evaluated in Marc-145 cells as previously described (Leng et al., 2017). Briefly, the rescued viruses were serially passaged in Marc-145 cells toward F5, and viral titers were determined by the presence of visible CPEs, and the results are recorded as $TCID_{50}$/100 μl using the Reed-Muench method. Marc-145 cell monolayers in 6-well plates were then infected with the rescued viruses at a multiplicity of infection (MOI) of 0.1. After 1 h of attachment at 37 °C with 5% CO_2 and gentle mixing every 10 min, the monolayers were washed three times with serum-free DMEM; 2 ml of 2% FBS DMEM was then added, and the plates were incubated for up to 4 days at 37 °C with 5% CO_2. Supernatants were collected every 12 h (performed in triplicate) post-infection. The growth kinetics of the parental and chimeric viruses were determined in Marc-145 cells in 96-well plates by the presence of visible CPEs, and the results are expressed as $TCID_{50}$/100 μl using the Reed-Muench method.

2.6. Statistical analysis

Significance among the growth kinetics of the rescued viruses was analyzed using Student's *t*-test in GraphPad Prism (version 5.0) software. Differences were considered significant at p values < 0.05 and extremely significant at p values < 0.01 and p values < 0.001.

3. Results

3.1. Genomic characteristics of Marc-145 cells un-adaptive wild-type PRRSVs

Since 2014, we have encountered in our lab numerous PRRSV strains incapable of being isolated from Marc-145 cells; interestingly, these PRRSV isolates were successfully isolated from PAMs. Moreover, the number of these PRRSV isolates has increased recently (Fig. 1A). Field HP-PRRSVs are well known to be readily isolated from both PAMs and Marc-145 cells. However, 10 field PRRSVs causing a 20%–30% mortality rate on pig farms were isolated from PAMs but not from Marc-145 cells. To explore which gene(s) determine(s) the change in adaptation of these PRRSVs, we sequenced their complete genomes and analyzed their genomic characteristics. In China, lineage 8 PRRSVs can be divided into seven sublineages (Gao et al., 2017). Phylogenetic analysis and sequence alignment showed that seven viruses belong to HP-PRRSV-related strains (lineage 8) (three of which belong to sublineage 8.3; another two are unclassified) and three to NADC30-like strains (lineage 1) (Fig. 1C). In addition, a 30-aa or 131-aa