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Stable expression of foreign gene in nonessential region of nonstructural protein 2 (nsp2) of porcine reproductive and respiratory syndrome virus: Applications for marker vaccine design

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ABSTRACT

The nonstructural protein 2 (nsp2) of porcine reproductive and respiratory syndrome virus (PRRSV) has been shown to be highly heterogeneous and variable among PRRSV strains and some sequences in the middle region of the nsp2 are not essential to viral replication. Recent studies have attempted to insert foreign genes in the nsp2 nonessential regions but the foreign genes were not stably expressed by recombinant viruses *in vitro*. In the present study, we first constructed an infectious cDNA clone with deletion of 75 nucleotides (25 amino acids) in the nsp2 region (rHuN4-F112-Δ508–532) of the attenuated vaccine virus HuN4-F112 derived from a highly pathogenic PRRSV HuN4 and then inserted a gene fragment encoding a immunodominant B-cell epitope (49 amino acids) of Newcastle disease virus (NDV) nucleoprotein (NP) in-frame into the deletion site. The viable recombinant virus was rescued from the full-length cDNA infectious clone *in vitro*. The engineered viruses rescued from the cDNA clone indicated that the deletions of 75 nucleotides and insertion of NDV NP gene in the nsp2 region did not affect viral replication; they had similar growth kinetics to its parental virus. The inserting gene could be expressed consistently when the recombinant virus was passaged up to twenty times in cell cultures as determined by immunofluorescence assay (IFA) and genomic sequencing. To investigate the potential application of the NDV NP gene-inserted PRRSV as a marker vaccine, piglets were immunized with the recombinant virus and then challenged with lethal dose of highly pathogenic PRRSV. The immunized piglets produced specific antibodies against both the NDV NP and PRRSV, and lacked antibodies against the deleted 25aa nsp2 epitope. After challenge, all immunized piglets were protected from clinical disease or death, while all piglets in control group died (5/5) by ten days post challenge. The results of the present study indicated that the recombinant PRRSV (rHuN4-F112-Δ508–532) could be used as a potential marker vaccine against PRRS.

1. Introduction

Since porcine reproductive and respiratory syndrome (PRRS) was initially identified in 1987 in the United States (Benfield et al., 1992; Keffaber, 1989) and in 1990 in Europe (Wensvoort et al., 1991), it has caused tremendous economic losses to the swine industry worldwide. The PRRS virus was isolated in 1991 in the Netherlands (Wensvoort et al., 1992b) and in 1992 in the United States (Benfield et al., 1992). The first two PRRSV isolates Lelystad and VR-2332 were respectively designated as the representative strains for the European (Type I) and North American (Type II) genotypes (Allende et al., 1999; Wensvoort et al., 1992a) according to their genome

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sequence difference. In China, PRRS was reported in 1995 (Guo et al., 1996). Later, a highly pathogenic type of PRRS (HP-PRRS) emerged in 2006 (Li et al., 2007; Tian et al., 2007; Tong et al., 2007). Since its emergence, HP-PRRS has been prevalent in most of swine herds in China (Han et al., 2007; Li et al., 2007; Tian et al., 2007; Tong et al., 2007, 2011; Zhou et al., 2008; Zhou et al., 2011).

The PRRSV genome contains ten open reading frames (ORF1a, ORF1b, ORF2, ORF2a, ORF3, ORF4, ORF5a, and ORF5–ORF7). ORF5a was a newly discovered (Firth et al., 2011; Johnson et al., 2011) reading frame. The ORF1a and ORF1b encode viral replicase polyproteins, which are directly translated upon viral entry and cleaved into 14 nonstructural proteins (nsp1α, nsp1β, nsp2 to nsp6, nsp7α, nsp7β, and nsp8 to nsp12) (Chen et al., 2010; Fang and Snijder, 2010; Snijder and Meulenberg, 1998). The nonstructural proteins participate in viral replication and transcription. Among the 14 nonstructural proteins, the nsp2 protein is the largest replicase protein of PRRSV (Fang and Snijder, 2010). The nsp2 region is significantly different in length between PRRSV type I and type II and it is even variable among strains of the same genotype. Genetic mutations or deletions always naturally occur in the middle or near the N-terminus of the nsp2 region in field strains (den Boon et al., 1995; Snijder et al., 1993, 1995; Zhou et al., 2008; Zhou et al., 2011). Previous studies have shown that HP-PRRSV strain has two unique discontinuous deletions of 30 amino acids in the middle of the nsp2 region (Tian et al., 2007; Tong et al., 2007).

Several nonessential regions for viral replication have been identified in PRRSV nsp2, and subsequently, some marker genes were successfully inserted to the nonessential region of PRRSV nsp2, such as green fluorescent protein (GFP) (Han et al., 2007; Fang et al., 2006, 2008), FLAG, EGFP and luciferase (Kim et al., 2007). The GFP-containing PRRSV genome reproduced viruses but GFP gene was not stable during passages of the recombinant viruses in cell cultures (Han et al., 2007), or mutations in GFP gene resulted in loss of fluorescence (Fang et al., 2006, 2008; Kim et al., 2007). In this study, 75 nucleotides were deleted from the nsp2 region of an attenuated HP-PRRSV and a NP gene fragment of Newcastle disease virus (NDV) was inserted into the deletion site by reverse genetic manipulation. The rescued recombinant PRRSV was evaluated for growth kinetics in cell cultures and for abilities to induce protection against virulent HP-PRRSV challenge and NDV specific antibody response in piglets. The production of antibodies to NDV NP and lack of antibody response to deleted 25aa of nsp2 in vaccinated pigs could serve as a serological marker for a modified live vaccine against HP-PRRS.

2. Materials and methods

2.1. Cells and viral strains

MARC-145 cells (African green monkey kidney epithelial cell line) were used to grow PRRSV. BHK-21 (Baby hamster kidney cells) was used for transfection. Both MARC-145 and BHK-21 cells were grown and maintained in Dulbecco's modified Eagle's high glucose medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA) at 37 °C in 5% CO_2. The HP-PRRSV HuN4 strain (GenBank accession no. EF635006) was isolated in 2006 from a piglet died of "high fever syndrome" and the attenuated vaccine virus HuN4-F112

Table 1
Oligonucleotide primers used in this study.

Primer[a]	Sequence[b]	Genomic position of amplified sequences[c]
F16(sp6)	5′-CCG*TTAATTAA***ATTTAGGTGACACTATA**GGATGACGTATAGGTGTT-3′	1–16
R2355	5′-GTGATGAACCTCGTCACCTTGTGCAGGG-3′	2355–2382
F2300	5′-CTTTGGGCAAGGACTCGGT-3′	2300–2319
R5914	5′-GATCCTGTGTGAACGCCGAC-3′	5914–5933
F5853	5′-CTTCTGCTTCACCGCGTGT-3′	5853–5871
R8825	5′-AAGAAGATTGGCGGCAAAC-3′	8825–8843
F8764	5′-GCAGGTGCCTTGAAGCTGAT-3′	8764–8783
R11910	5′-CTCATGCTGATGGCATTAGC-3′	11,910–11,929
F11851	5′-AGGACTGGGAGGATTACAAT-3′	11,851–11,870
R14670	5′-CGGACGACAAACGCGTGGTTAT-3′	14,670–14,691
F14668	5′-TGATAACCACGCGTTTGTCGTC-3′	14,668–14,689
R15313	5′-TATAGCGGCCGC (T)32AATTACGG-3′	15,313–15,320
FΔ508–532	5′-AGCCCGTACTTATGCCCGCGACAACGCTGACGCACCAGGA-3′	2837–2858, 2932–2951
RΔ508–532	5′-TCCTGGTGCGTCAGCGTTGTCGCGGGCATAAGTACGGGCT-3′	2837–2858, 2932–2951
F-NDV NP	5′-CGCGAATTCATGTCTTCCGTATTTGATGAGT-3′	122–143
R-NDV NP	5′-ATACTCGAGTCAATACCCCCAGTCGGTGT-3′	1572–1591
FΔ508–532-NP49	5′-AGCCCGTACTTATGCCCGCGGGGGATGGGGAGACCCAAT-3′	2837–2951-NP49
RΔ508–532-NP49	5′-TCCTGGTGCGTCAGCGTTGTATACCCCCAGTCGGTGTCGT-3′	2837–2951-NP49
F-NDV NP441–489	5′-TTAGAATTCGGGGATGGGGAGACCCAAT-3′	1442–1460
R-NDV NP441–489	5′-ATACTCGAGATACCCCCAGTCGGTGTCGT-3′	1569–1588
R2701	5′-GCTCCGCGCAGGAAGGT-3′	2701–2717
F3192	5′-GGAGATGCCCACTGCAA-3′	3175–3192
R14320	5′-CACAGCTCCACAGAAGGTGC-3′	14,320–14,339
F14752	5′-TAACAGCTTTTCTGCCACCC-3′	14,752–14,771

[a] Forward primers are preceded by F before the designator, and reverse primers are indicated with R.
[b] SP6 polymerase promoter is shown in bold. Restriction enzyme sites are indicated in italic.
[c] Unless otherwise noted, genome position of PRRSV is based on GenBank accession No. EF635006 and genome position of NDV is based on GenBank accession No. AY845400.