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HP-PRRSV is attenuated by de-optimization of codon pair bias in its RNA-dependent RNA polymerase nsp9 gene

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ABSTRACT

There is an urgent need to develop new vaccines against highly pathogenic PRRS virus (HP-PRRSV) variant in China. The actual use of each codon pairs is more or less frequent than that of the statistical prediction and codon pair bias (CPB) usage affects gene translation. We "shuffled" the existing codons in HP-PRRSV genes GP5, M, nsp2 and nsp9, so that the CPB of these genes could be more negative. De-optimization of nsp9, the RNA-dependent RNA polymerase, significantly decreased PRRSV replication in porcine alveolar macrophages (PAMs). *In vitro* study showed that HV-nsp9^{min} and HV-nsp29^{min} were remarkably attenuated in PAMs, and inoculation of pigs with 2 ml$*10^{5.0}$ $TCID_{50}$/ml of HV-nsp9^{min} or HV-nsp29^{min} did not cause PRRS. Importantly, pigs immunized with HV-nsp29^{min} were fully protected against different HP-PRRSV strains' lethal challenges. Our results imply that the CPB de-optimized HV-nsp29^{min} has the potential to be used as a live vaccine candidate against HP-PRRSV.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important diseases in the swine industry (Neumann et al., 2005; Nieuwenhuis et al., 2012; Tian et al., 2007). It is estimated that the losses associated with PRRS are approximately $664 million per year in the United States alone (Neumann et al., 2005). Most recently, there have been devastating outbreaks of atypical PRRS in China and neighboring countries with a mortality of 20 to 100% (Li et al., 2007; Zhou et al., 2008), which is caused by a highly pathogenic PRRSV (HP-PRRSV) variant with an unique molecular hallmark, namely a discontinuous deletion of 30 amino acids in nonstructural protein 2 (nsp2). Currently, vaccination is generally used to control atypical PRRS. However, the available vaccines for HP-PRRSV are known to have drawbacks.

The etiological agent, PRRS virus (PRRSV), is a ~15.4 kb positive-sense RNA virus belonging to the *family Arteriviridae*, the *order Nidovirales* (Gorbalenya et al., 2006). Comparative genome sequence analysis has revealed that PRRSV has two distinct genotypes, type 1 (European type) and type 2 (North American type) (Benfield et al., 1992; Nelsen et al., 1999). The PRRSV genome consists of at least 10 overlapping open reading frames (ORFs): ORFs 1a, 1b, 2a, 2b, 3, 4, 5a, 5, 6, 7 (Fang and Snijder, 2010; Firth et al., 2011; Johnson et al., 2011; Li et al., 2014; Oh and Lee, 2012; Snijder and Meulenberg, 1998). ORF1a and ORF1b encode replicative enzymes (Fang and Snijder, 2010), of which nsp2 is the largest PRRSV protein and its central region is highly variable with deletions and insertions (Fang et al., 2004; Kim et al., 2010; Yoshii et al., 2008). The key enzymes for PRRSV RNA synthesis are encoded in ORF1b, in particular the viral RNA-dependent RNA polymerase (RdRp, nsp9), which is essential for both genome replication and the synthesis of a nested set of subgenomic (sg) mRNAs (Fang and Snijder, 2010). The GP5 and M proteins, which are encoded by ORF5 and ORF6, respectively, are the major structural proteins of PRRSV. It has been shown that one of the major virulence determinants of PRRSV is located in GP5 (Kwon et al., 2008). As a non-glycosylated membrane protein, M is closely associated with the function of GP5 and likely plays a key role in virus assembly and budding.

The utilization of codon pair in protein coding sequences is highly biased. Some pairs of codons are overrepresented, while other pairs are underrepresented compared to the frequency

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expected from the usage of the individual codons of these pairs. This phenomenon is termed as codon pair bias (CPB) (Gutman and Hatfield, 1989). Codon-pair bias has been found in every species examined, and it can be quantified statistically (Moura et al., 2007). Although it is not clear how codon pair preference is formed (Buchan et al., 2006), the codon pair usage in an open reading frame can influence the gene expression (Irwin et al., 1995). It has been shown that synthesizing novel poliovirus and influenza virus to contain underrepresented codon pairs while exactly preserve the codon usage and amino acid sequence, can dramatically attenuate the virus (Coleman et al., 2008, 2011; Mueller et al., 2010; Yang et al., 2013). Recently, a report showed that deoptimization of the major envelope GP5 gene attenuated PRRSV (Ni et al., 2014).

To attenuate the HP-PRRSV strain HV, we redesigned large parts of the coding regions of nsp2, nsp9, GP5 and M using rare codon pairs by a specific de-optimization computer program (Coleman et al., 2008). The program can rearrange synonymous codons to yield rare codon pairs while not altering amino acid sequence. Using chemical synthesis and reverse genetics we constructed and rescued the recombinant HP-PRRSV virus HV-nsp2^{min}, HV-nsp9^{min}, and HV-nsp29^{min}. To be compared with their parental strain HV, the recombinant viruses, especially HV-nsp9^{min} and HV-nsp29^{min} replicated significantly slower *in vitro*. Most importantly, HV-nsp9^{min} and HV-nsp29^{min} were attenuated *in vivo* and pigs vaccinated with HV-nsp29^{min} were completely protected from the lethal challenge with HP-PRRSV strains.

Results

The CPB in pig genome and construction of codon pair de-optimized highly pathogenic PRRS virus

To attenuate the highly pathogenic PRRS virus HV strain by de-optimization of the virus gene codon pair usage, we first analyzed the codon pair usage in the pig genome using the algorithm described before (Coleman et al., 2008). Our analysis revealed that there was a normal distribution between −0.2 to 0.3 for the CPB value of the pig genes (Fig. 1). We then re-coded part coding regions for HV nsp2, nsp9, GP5, and M genes using codon pairs which are under-represented relative to the pig genome through shuffling the synonymous codons without altering amino acid sequence. This resulted in dozens or even hundreds of silent mutations (Table 1), and the CPB values of the re-coded gene sequences were much lower than that of normal pig genes (from −0.04472 to −0.40141 for GP5, −0.04297 to −0.40440 for M, from −0.03984 to −0.40700 for nsp2, and −0.00548 to −0.20000 or −0.40210 for nsp9) (Fig. 1 and Table 1).

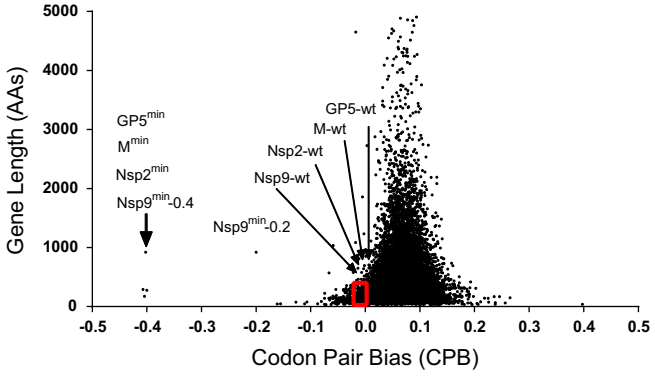

Fig. 1. Calculated codon pair bias (CPB) score for all 14,724 swine genes. Each dot represents the calculated CPB score of a gene plotted against its amino acid length. Underrepresented codon pairs yield negative scores. The CPB of various PRRSV gene segments are indicated by arrows.

The characteristics of the recoded gene segments and the changes of their codon pair bias are summarized in Table 1.

To generate HV virus carrying the de-optimized gene segments, we synthesized and incorporated the rearranged segments into the full-length infectious cDNA construct of HV virus, and the resulted plasmids were then transfected into 293FT cells. At 48 h after transfection, cell culture supernatants were detected for viable virus. Only HV with de-optimized nsp2 (designated as HV-nsp2^{min}) and HV with de-optimized nsp9 (CPB = −0.2) (designated as HV-nsp9^{min} −0.2) yielded viable viruses (Fig. S1), while the other three constructs did not produce any viable virus even after three blind passages of the supernatants from transfected cells. In order to get additional effects of the codon pair de-optimization, we constructed HV-nsp29^{min} plasmid with the de-optimized nsp2 and de-optimized nsp9 (CPB = −0.2), and yielded the de-optimized recombinant virus HV-nsp29^{min} (Fig. S1). For convenience, the virus HV-nsp9^{min} −0.2 was abbreviated as HV-nsp9^{min} in the following studies.

Decreased fitness of the codon pair de-optimized recombinant virus in PAMs

To evaluate and compare the *in vitro* growth characteristics between the recombinant virus HV-nsp2^{min}, HV-nsp9^{min}, HV-nsp29^{min}, and their parental virus HV, we examined their growth kinetics in PAMs infected with an moi of 0.01. These de-optimized viruses did not replicate as well as the wild-type HV (HV-wt). As shown in Fig. 2A, there were hardly any living PAMs existed at 84 h post-infection (hpi) with wild-type HV. However, there was no obvious cytopathic effect observed in PAMs infected with either HV-nsp9^{min} or HV-nsp29^{min} (Fig. 2A). The cytopathic effect induced by the mutant virus HV-nsp2^{min} was only slightly reduced with respect to the wild-type HV (Fig. 2A). These mutated viruses had similar growth curves with the wild-type HV, but they grew much slower than the wild-type HV, and the titers of the de-optimized viruses were lower compared to the wild-type HV at all the time points examined during the replication (Fig. 2B). The titer of HV-nsp2^{min} at 84 hpi was decreased 4 folds compared to wild-type HV, whereas the titer of HV-nsp9^{min} at 84 hpi was much lower by orders of magnitude, up to a factor of 1659. The recombinant virus HV-nsp29^{min} containing both nsp2^{min} and nsp9^{min} replicated as much slowly as HV-nsp9^{min}, suggesting that de-optimization of PRRSV RNA-dependent RNA polymerase nsp9 has much severer effect on virus replication. These results corresponded well with nsp2, nsp9, and GP5 protein and mRNA expression levels analyzed by western blot (Fig. 2C) and RT-PCR (Fig. 2D). Previous studies had hypothesized that attenuation of virus through codon pair bias maybe result from the down-regulation of protein synthesis (Coleman et al., 2011). We therefore analyzed the expression of the wild-type and mutated nsp2 and nsp9 genes cloned into pCMV-myc vector in porcine cell line CRL-2843 using western blot. As shown in Fig. 2E, protein levels of both nsp2^{min} and nsp9^{min} were significantly down-regulated compared to their parental wild-type genes. However, the mRNA expressions of the mutated genes were even slightly higher when compared to their parental wilt-type genes (Fig. 2F). Collectively, these results suggest that codon pair de-optimization of viral genes decreases their protein expressions, leading to the defect in replicative capacity of the mutational viruses relative to their parental virus.

HV-nsp29^{min} and HV-nsp9^{min} was attenuated in pigs

Given that HV-nsp29^{min} was highly attenuated in PAMs, we next investigated its growth phenotype and pathogenesis in pigs to examine whether it was also attenuated *in vivo*. Two groups of 4-week-old healthy conventional pigs were inoculated with either wild-type HV