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Expression of antigenic epitopes of porcine reproductive and respiratory syndrome virus (PRRSV) in a modified live-attenuated porcine circovirus type 2 (PCV2) vaccine virus (PCV1-2a) as a potential bivalent vaccine against both PCV2 and PRRSV

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ABSTRACT

Co-infection of pigs in the field with porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) is common and poses a major concern in effective control of PCV2 and PRRSV. We previously demonstrated that insertion of foreign epitope tags in the C-terminus of PCV2 ORF2 produced infectious virions that elicited humoral immune responses against both PCV2 capsid and inserted epitope tags. In this study, we aimed to determine whether the non-pathogenic chimeric virus PCV1-2a, which is the basis for the licensed PCV2 vaccine Fostera™ PCV, can express PRRSV antigenic epitopes, thus generating dual immunity as a potential bivalent vaccine against both PCV2 and PPRSV. Four different linear B-cell antigenic epitopes of PRRSV were inserted into the C-terminus of the capsid gene of the PCV1-2a vaccine virus. We showed that insertion of 12 (PRRSV-GP2 epitope II, PRRSV-GP3 epitope I, and PRRSV-GP5 epitope I), and 14 (PRRSV-GP5 epitope IV) amino acid residues did not impair the replication of the resulting PCV1-2a-PRRSV$_{EPI}$ chimeric viruses *in vitro*. The four chimeric PCV1-2a viruses expressing PRRSV B-cell linear epitopes were successfully rescued and characterized. An immunogenicity study in pigs revealed that two of the four chimeric viruses, PCV1-2a-PRRSV$_{EPI}$GP3IG and PCV1-2a-PRRSVEPI$_{EPI}$GP5IV, elicited neutralizing antibodies against PRRSV VR2385 as well as PCV2 (strains PCV2a, PCV2b, and mPCV2b). The results have important implications for exploring the potential use of PCV1-2a vaccine virus as a live virus vector to develop bivalent MLVs against both PCV2 and PRRSV.

1. Introduction

Porcine circovirus type 2 (PCV2) is the causative agent of porcine circovirus-associated disease (PCVAD) (Allan et al., 1999; Chae, 2005; Ge et al., 2012). Among the most common clinical presentations associated with PCVAD is post-weaning multisystemic wasting syndrome (PMWS) (Kennedy et al., 2000). PCV2 is also considered to be a part of the porcine respiratory disease complex (PRDC) (Kim et al., 2003). Numerous reports have shown the importance of PCV2 in the development of PCVAD, however PCVAD has rarely been reproduced experimentally by PCV2 infection alone (Ge et al., 2012; Puvanendiran et al., 2011). Nevertheless, clinical PCVAD has been experimentally reproduced in PCV2 co-infection models with porcine parvovirus infection (Allan et al., 1999; Kennedy et al., 2000; Opriessnig et al., 2004a), *Mycoplasma hyopneumoniae* (Opriessnig et al., 2006; Opriessnig et al., 2004b), or porcine reproductive and respiratory syndrome virus (PRRSV) (Allan et al., 2000; Opriessnig et al., 2008).

Porcine reproductive and respiratory syndrome (PRRS) is caused by PRRSV (Halbur et al., 1995; Meng et al., 1995). The major clinical manifestations of PRRS are reproductive failures, which are characterized by abortions and high percentages of mummified fetuses (Karniychuk and Nauwynck, 2013). Additional clinical manifestations of PRRS include respiratory problems in nursery and

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growing-finishing pigs, growth reduction, poor performance, and high mortality rate (Halbur et al., 1996; Mengeling et al., 1998). Both PRRSV and PCV2 have caused devastating diseases in swine industry worldwide, resulting in immense economic losses. Co-infection of pigs with PCV2 and PRRSV in the field are common in swine production worldwide. The prevalence of natural PRRSV and PCV2 co-infection in swine herds varies from 20% in Canada, 48% in Spain and 60% in the United States (Pallarés et al., 2002; Park et al., 2014; Pogranichniy et al., 2002; Quintana et al., 2001; Rovira et al., 2002). Therefore, prevention and control of PRRSV and PCV2 co-infection have been a priority for the global swine industry.

These two economically-important swine diseases are caused by completely unrelated viruses. PCV2 belongs to the family *Circoviridae* [16], which is a non-enveloped, single-stranded circular DNA virus of approximately 1.7 kb in genome size with 11 predicted potential open reading frames (ORFs) (Hamel et al., 1998). PCV2b is currently the predominant subtype infecting pigs worldwide, although PCV2a subtype is also prevalent in swine herds (An et al., 2007; Shen et al., 2012; Trible and Rowland, 2012). The two major ORFs of PCV2 are ORF1 which encodes the full-length replicase (Rep) of 314 amino acids (aa) and a truncated, spliced Rep' protein (178 aa), and ORF2 which encodes the viral capsid protein (233 aa). PRRSV is a single-stranded, positive-sense RNA virus of approximately 15 kb in genome size that belongs to the family *Arteriviridae.* The genome of PRRSV consists of nine ORFs encoding nonstructural proteins (ORFs 1a, 1b) and structural proteins (ORFs 2a, 2b, 3, 4, 5, 6, and 7). There exist two distinct genotypes of PRRSV: North American (type 2) and European (type 1) (Halbur et al., 1995; Meng et al., 1995; Nelson et al., 1993; Wootton et al., 2000). The two genotypes share approximately 60% nucleotide sequence identity and are antigenically different (Allende et al., 1999; Lunney et al., 2010; Nelsen et al., 1999; Plagemann et al., 2002).

Both PRRSV and PCV2 are capable of tolerating mutations and insertion of foreign nucleic acids sequences without affecting their infectivity *in vivo* (Beach et al., 2011; Huang et al., 2014; Pei et al. 2009). PCV2 can tolerate an insertion of up to 27 aa residues of foreign antigenic epitopes into the C-terminus of the ORF2 without impairing its infectivity *in vivo* (Beach et al., 2011). More recently, insertion of a foot and mouth disease virus (FMDV) VP1 epitope in PCV2 resulted in infectious viruses that elicited dual immunity against PCV2 and FMDV (Huang et al., 2014).

Several commercial vaccines against PCV2 are currently available, including inactivated whole virus, inactivated chimeric PCV1-2a virus, and subunit vaccines (Beach and Meng, 2012). Currently, a licensed modified live-attenuated vaccine (MLV) against PCV2 is still lacking. It has been shown that the replication factors of the non-pathogenic PCV1 and the PCVAD-associated PCV2 are interchangeable (Beach et al., 2010a; Mankertz et al., 2003). A candidate MLV against PCV2 has been developed using a chimeric virus approach, in which the ORF2 capsid gene of pathogenic PCV2 was inserted into the backbone of the non-pathogenic PCV1. The resulting chimeric PCV1-2a virus is non-pathogenic but elicits protective immunity against PCV2 in pigs (Fenaux et al., 2004) and was the basis for the first USDA-fully licensed vaccine against PCV2. Similarly, another chimeric virus PCV1-2b, which the ORF2 capsid gene of PCV2b subtype was inserted in the backbone of PCV1, has been demonstrated to confer cross-protection against different PCV2 subtypes (Beach et al., 2010b). Due to PRRSV immune evasion and high genetic diversity among PRRSV strains, the current PRRSV vaccines only confer protection against closely-related strains but not against heterologous strains. Currently there are two type of commercial vaccine available, MLV and inactivated killed vaccines (KV) (Hu and Zhang, 2012; Zuckermann et al., 2007).

Since PCV2 and PRRSV co-infections are very common in swine herds worldwide, it would be of great interest to develop a bivalent MLV vaccine that can protect pigs against both viruses. Therefore, it is logical to explore the potential use of the non-pathogenic chimeric PCV1-2a vaccine virus, which is the basis of the current commercial killed vaccine Fostera™ PCV, as a live virus vector to express PRRSV antigenic epitopes for use as a potential PCV2-PRRSV bivalent MLV vaccine.

2. Material and methods

2.1. Construction of chimeric PCV1-2a-PRRSV$_{EPI}$ (epitope) infectious DNA clones

Four different antigenic epitopes of PRRSV strain VR2385, including GP2 epitope II (aa 39–51, ASPSHVGWWSFA), GP3 epitope I (aa 61–72, QAAAEAYEPGRS), GP5 epitope I (aa 35–46, SSSNLQLIYNLT), and GP5 epitope IV (aa 186–200, TPVTRVSAEQWGRP), were cloned individually in frame into the C-terminus of the ORF2 capsid gene of the chimeric PCV1-2a vaccine virus previously developed in our laboratory (Fenaux et al., 2004). Chimeras were constructed by overlapping extension PCR following a method described previously (Beach et al., 2011). Briefly, two amplicons of 212 bp and 1800 bp with an overhanging complementary region containing different PRRSV antigenic epitopes (Table 1) and a *Sac*II restriction site were constructed by overlapping extension PCR using the PCV1-2a infectious clone plasmid pBSK-PCV1-2a (Fenaux et al., 2004) as the template. A second round of fusion PCR was used to assemble the two amplicons. The PCR product was digested with *Sac*II (New England Biolabs) and ligated into pBluescript II SK (+) (pBSK+) (Stratagene) to generate each of the chimeric PCV1-2a-PRRSV$_{EPI}$ DNA clones (Fig. 1). Recombinant plasmids containing the respective insert were transformed into the alpha-select strain of *Escherichia coli* (Bioline). Positive clones were selected *via* blue–white screening and the insertion of each specific PRRSV antigenic epitope was confirmed by sequencing. Chimeric PCV1-2a-PRRSV$_{EPI}$ viruses were rescued by transfection of concatemerized genomic DNA of each chimera into the PCV1-free PK-15 cells as previously described (Fenaux et al., 2003). Infectious viruses were harvested by freezing and thawing the cell cultures three times and were stored at −80 °C for further use.

2.2. Infectivity, PRRSV antigenic epitope expression, and comparative growth kinetics of chimeric PCV1-2a-PRRSV$_{EPI}$ viruses

To determine the infectivity of the PCV1-2a-PRRSV$_{EPI}$ chimeric viruses, PK-15 cells were seeded at a concentration of 2×10^5 cells/well in 48 well plates and infected at 40–50% confluency with 100 μL of 1:10 serial dilutions of each virus stock. After 72 h, the cells were fixed, and virus infectivity was detected by immunofluorescence assay (IFA) using 1:1000 dilution of a mouse anti-PCV2-Cap specific monoclonal antibody (mAb) (RTI, Brookings, SD), followed by fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG-FITC (KPL, Kirkegaard & Perry Laboratories, Inc.). The positive fluorescence signals were detected using a fluorescent microscope. Expression of the PRRSV-specific antigenic epitopes was detected by IFA using custom polyclonal antibodies (Biomatik) against each of the PRRSV synthetic antigenic epitope, followed by a secondary goat anti-rabbit IgG (DyLight 550; emission spectrum peak wavelength of approximately 576 ± 4 nm; Thermo Scientific). Serial ten-fold dilutions of each virus stock were performed in order to determine the tissue culture infectious dose 50 ($TCID_{50}$) according to the method described by Reed and Muench (1938).

To compare the replication and growth kinetics of the PCV1-2a-PRRSV$_{EPI}$ chimeric viruses, and its parental PCV1-2a vaccine virus, six well plates were seeded with PK15 cells and infected at a