Original Article

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Phenotypic and genotypic analyses of an attenuated porcine reproductive and respiratory syndrome virus strain after serial passages in cultured porcine alveolar macrophages

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The porcine reproductive and respiratory syndrome virus (PRRSV) is a globally ubiquitous swine viral pathogen that causes major economic losses worldwide. We previously reported an over-attenuated phenotype of cell-adapted PRRSV strain CA-2-P100 *in vivo*. In the present study, CA-2-P100 was serially propagated in cultured porcine alveolar macrophage (PAM) cells for up to 20 passages to obtain the derivative strain CA-2-MP120. Animal inoculation studies revealed that both CA-2-P100 and CA-2-MP120 had decreased virulence, eliciting weight gains, body temperatures, and histopathologic lesions similar to those in the negative control group. However, compared to CA-2-P100 infection, CA-2-MP120 yielded consistently higher viremia kinetics and enhanced antibody responses in pigs. All pigs inoculated with CA-2-MP120 developed viremia and seroconverted to PRRSV. During 20 passages in PAM cells, CA-2-MP120 acquired 15 amino acid changes that were mostly distributed in nsp2 and minor structural protein-coding regions. Among these changes, 6 mutations represented reversions to the sequences of the reference CA-2 and parental CA-2-P20 strains. These genetic drifts may be hypothetical molecular markers associated with PRRSV macrophage tropism and virulence. Our results indicate that the PAM-passaged CA-2-MP120 strain is a potential candidate for developing a live, attenuated PRRSV vaccine.

Keywords: attenuated vaccines, macrophage tropism, porcine reproductive and respiratory syndrome virus, virulence, whole genome sequencing

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure in pregnant sows and acute or chronic respiratory illnesses in pigs of all ages. PRRS virus (PRRSV), the etiological agent of PRRS, is a small, enveloped, single-stranded, positive-sense RNA virus that is a member of the genus *Porarterivirus* in the family *Arteriviridae* placed within the family *Coronaviridae* in the order *Nidovirales* [1,29]. The PRRSV genome is ~15 kb long with a 5′ cap structure and a 3′ polyadenylated tail and contains at least 10 open reading frames (ORFs) flanked by untranslated regions (UTRs) at both the 5′ and 3′ termini [29]. The first two ORFs,

ORF1a and 1b, in the 5′-proximal three-quarters of the genome encode nonstructural proteins (nsps), while the remaining ORFs in the 3′-proximal part of the genome encode eight structural proteins [14,29].

PRRSV emerged nearly simultaneously in the United States and Europe in the late 1980s and has since become omnipresent worldwide [15]. Despite tremendous efforts invested in controlling PRRSV, the virus is now recognized as the most globally notorious and widespread porcine viral pathogen and is a major economic burden on the world swine industry [13]. A hallmark of PRRSV is the high degree of genetic and antigenic drift due to a rapid mutation rate. Thus, PRRSV has been divided into two species: PRRSV-1 (formerly European

genotype 1) and PRRSV-2 (formerly North American genotype 2), which share approximately 60% nucleotide (nt) identity at the genome level [1,26]. Although the two species initially represented the topotype of each respective continent, they have now emerged and re-emerged worldwide [25,34]. PRRSV continues to undergo swift evolution with clinical variations of the disease showing nt sequence divergence of up to 20% among isolates within each species [8,11]. This genetic heterogeneity results in substantial biological and pathogenic diversity among PRRSV field isolates, which is one of the main barriers to developing more effective vaccines to combat PRRS.

In Korea, the first case of PRRSV-2 infection was described in 1993 [19], and the disease has since become a significant problem for swine production, leading to immense financial losses. Emergence of PRRSV-1 in Korea was reported in 2006, and the intermingling of the two species has since occurred in Korea, leading to critical issues in PRRSV management [17,18,25]. Even though the presence of a highly pathogenic PRRSV that appeared in China and its neighboring countries has never been identified, at least 4 different lineages of PRRSV-2 circulate in Korea [16,28]. In particular, PRRSV-2 lineage 1 that includes the virulent MN184 and relative strains, which has spread across the mid-western US since 2000 [12], has severely affected the pork industry in Korea since the early 2010s [6]. Compared to the PRRSV-2 prototype VR-2332, the virulent lineage 1 strains contain a discontinuous 111-1-19 deletion (DEL) of 131 amino acids (aa) within the middle hypervariable 2 (HV2) region of nsp2 (nsp2 111-1-19 DEL) [4,12].

Although considerable research investment has been provided to decipher PRRSV biology and develop measures for its management, critical information for the eradication of the virus is still lacking. An important strategy to control PRRS is to operate a system that can monitor the circulation of the virus in pig-producing regions. Additionally, it is necessary to explore safe and efficient vaccines using epizootic strains, if possible. In our previous study, the virulent Korean PRRSV nsp2 DEL strain CA-2 was passaged 100 times in cultures of MARC-145 cells for viral attenuation. We found that strain CA-2-P100 (100th passage of CA-2) exhibited an attenuated phenotype in inoculated pigs and had numerous aa mutations distributed throughout its genome. However, some pigs challenged with CA-2-P100 remained viremia-negative and seronegative to PRRSV throughout the trial, implying that the virus might be over-attenuated [22]. Since PRRSV is a pathogenic macrophage-tropic arterivirus of swine, sequentially passaging PRRSV over 100 times in a non-host cell line may have caused it to lose its tropism to porcine alveolar macrophages (PAMs), which are targeted by the virus during infection in the natural host. In the present study, CA-2-P100, a high-passage derivative of CA-2 produced by serial passages in

MARC-145 cells, was additionally passaged 20 times in immortalized PAMs. The aim was to create appropriate conditions for the virus to revert to a macrophage-tropic phenotype that may be suitable for developing an MLV vaccine. We evaluated the virulence and immunogenicity of the strain CA-2-MP120 (20th passage of CA-2-P100 in a PAM cell line) in the natural host. In addition, the complete genome sequences of PAM-passaged derivatives of CA-2-P100 were determined to illuminate the potential relationships between PRRSV genetic mutations and virulence.

Materials and Methods

Cells and virus strains

PAM-KNU (an immortalized PAM cell line) and MARC-145 cells were cultured and maintained as described previously [22,27]. The previously reported PRRSV CA-2 strain was plaque-purified and propagated in MARC-145 or PAM-pCD163 cells [6,22]. The high-passage derivative of CA-2, namely CA-2-P100, was obtained by continuous passaging for 100 times in MARC-145 cells, as previously described [22]. CA-2-P100 was serially passaged in PAM-KNU cells as described elsewhere [23,27].

Animal inoculation studies

In vivo swine infection experiments described herein were performed at the Choongang Vaccine Laboratory Animal Facility under the guidelines established by its Institutional Animal Care and Use Committee (IACUC No. 150128-02). Twenty-four 3-week-old crossbred pigs (Great Yorkshire × Dutch Landrace) were obtained from a conventional pig farm with a good health record and randomly divided into four groups of six piglets. All pigs were tested for absence of antibodies to PRRSV, porcine circovirus 2, and swine influenza virus. The pigs were allowed to acclimate for one week and then inoculated intratracheally with 10^4 $TCID_{50}$/mL ($TCID_{50}$, 50% tissue culture infectious dose) of parental CA-2-P20 (Group 1), CA-2-P100 (Group 2), CA-2-MP120 (Group 3), or culture medium as a placebo (control group). For intratracheal inoculation, all pigs were anesthetized by intravenous injection with a mixture of 7 mg/kg tiletamine/zolazepam (Zoletil 50; Virbac Laboratory, France) and 1 mg/kg xylazine (Rompun; Bayer Korea, Korea), and restrained with the head in a vertical position. A tracheal tube was advanced through the larynx deep into the bronchi via laryngoscope, and 7 mL of viral suspension or placebo were slowly administered. Rectal temperatures were measured twice daily, and clinical parameters were monitored daily throughout the experiment. Pigs were weighed at 0, 3, 6, 8, 14, 21, 28, and 35 days post-inoculation (dpi), and blood samples were collected at the same times. All animals were necropsied at 35 dpi and subjected to post-mortem examinations as described previously [10,22,24].

Viremia and serology

PRRSV levels in serum samples were assessed by virus titration using MARC-145 or PAM-pCD163 cells as described previously [6,22]. PRRSV-specific antibodies were detected by using a commercial PRRSV antibody enzyme-linked immunosorbent assay (ELISA) kit (HerdChek PRRS X3; IDEXX Laboratories, USA), according to the manufacturer's protocols, and a serum neutralization test as described previously [22].

Nucleotide sequence analysis

The full-length genomic sequences of CA-2-P100 strains at the 10th and 20th passages in PAM-KNU cells, designated CA-2-MP110 and CA-2-MP120, were determined by next-generation sequencing (NGS) technology as previously described [21,22]. The 5′ and 3′ ends of the genomes of the PAM cell-passaged CA-2-P100 strains were determined by a rapid amplification of cDNA ends technique as previously described [6]. The complete genomic sequences of the CA-2-P100 derivatives (CA-2-MP110 and -MP120) were deposited in the GenBank database (National Center for Biotechnology Information, USA) under accession No. KY363991 and KY363992, respectively. In addition, the unique aa substitutions identified in CA-2-MP110 or CA-2-MP120 were verified by applying the traditional Sanger sequencing method as previously described [6].

Statistical analysis

The significance of variability among the groups was determined by performing one-way analysis of variance followed by Tukey's *post hoc* test for multiple comparisons. All statistical analyses were conducted using Prism software (ver. 5.0; GraphPad, USA). Differences between groups were considered statistically significant at p values less than 0.05 ($p < 0.05$).

Results

Clinical assessments of pigs post-inoculation

In the current study, we serially passaged the previously MARC-145-adapted CA-2-P100 strain of PRRSV in cultured PAM cells. The strain obtained after the 20th passage of CA-2-P100 in PAM cells, which was designated as CA-2-MP120, was selected for subsequent phenotypic and genotypic studies. None of the pigs in the control group exhibited typical clinical signs of PRRS throughout the study. Half of the pigs in Group 1 transiently had moderate clinical symptoms such as dyspnea and anorexia between 3 to 10 dpi. Except for these animals, the remaining virus-inoculated pigs in Groups 1, 2, and 3 did not develop archetypal clinical parameters compared to the mock-infected control group.

The mean rectal temperatures in Group 1 indicated a persistent moderate fever (40.1°C–40.6°C) and were statistically higher than those of the control group between 7 and 14 dpi (panel A in Fig. 1). Interestingly, one of the pigs in Group 1 that exhibited mild clinical signs occasionally had a high fever (≥ 41°C) at 7, 10, and 14 dpi. In contrast, the rectal temperatures of the pigs in Group 2 were comparable to those of the control group throughout the study. Likewise, the overall febrile response of pigs in Group 3 was similar to those of the Group 2 and control animals for the entire experiment.

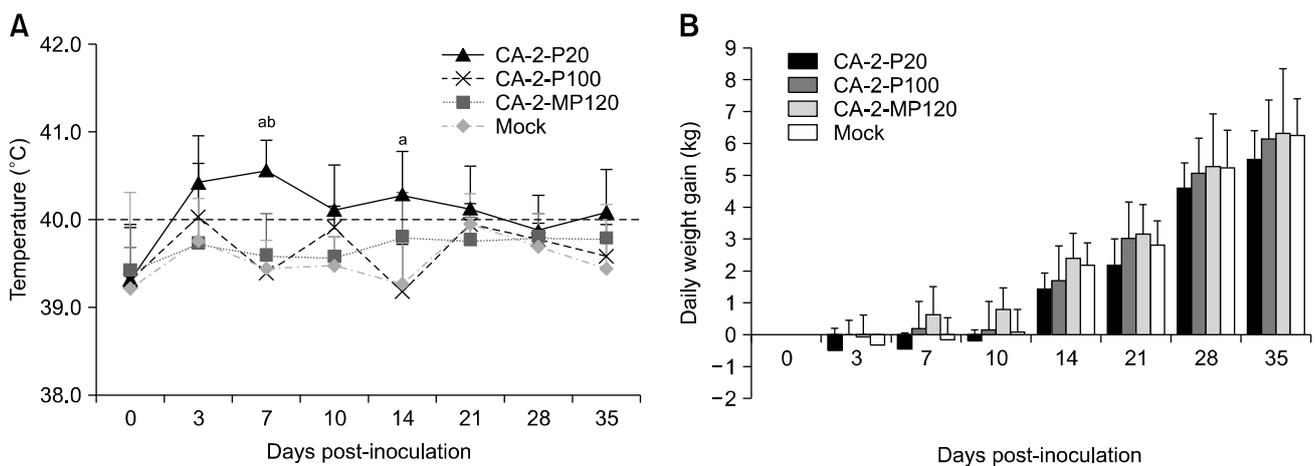There was no statistically significant difference in weight

Fig. 1. Average body temperature (A) and average daily weight gain (B) of pigs after challenge with the CA-2-P20, CA-2-P100, or CA-2-MP120 strains of porcine reproductive and respiratory syndrome virus or mock challenge with culture medium. Rectal temperatures equal to or greater than 40°C were defined as fever. Error bars represent SD. "a" denotes a significant difference ($p < 0.05$) between the CA-2-P20 and CA-2-P100 groups; "b" denotes a significant difference ($p < 0.05$) between the CA-2-P20 and CA-2-MP120 groups.

among the four groups throughout the experiment (panel B in Fig. 1). Animals in Group 1 exhibited a lower average daily weight gain (ADG) than the non-infected pigs, starting at 14 dpi. In contrast, pigs in Groups 2 and 3 gained weight in a manner similar to that of the control group. The largest difference in ADG between Groups 1 and 3 was approximately 1 kg from 10 to 21 dpi. These results demonstrated that the PAM-passaged CA-2-MP120 strain-infected pigs exhibited normal weight gain with no significant difference from the weight gains of the CA-2-P100-infected and control animals.

Viremia and antibody responses

All virus-challenged animals had viremia at different times post-inoculation, whereas the control group pigs remained viremia-negative throughout the experiment (panel A in Fig. 2). At 3 dpi, all animals (6/6) in Group 1 developed viremia, which was maintained until 21 dpi. In addition, viremia endured in two pigs from Group 1 until 28 dpi. Five of six pigs infected with CA-2-P100 (Group 2) intermittently experienced viremia for irregular periods, whereas the remaining Group 2 pig had no viremia throughout the experiment. Among the infected pigs from Group 2, two pigs were viremic only at 14 or 35 dpi, one animal was viremic between 21 and 35 dpi, and the remaining two pigs developed viremia by 7 or 10 dpi. Interestingly, the last instance exhibited transient viremia kinetics, since it was undetectable at 14 dpi but detectable between 21 to 28 dpi. In contrast, all pigs inoculated with CA-2-MP120 (Group 3) developed viremia at 3 dpi (4/6) or 7 dpi (2/6), and the virus endured in their sera until 28 dpi. The viremia duration in Group 3 was comparable to that observed in Group 1, even though the time of onset and the degree of viremia by 10 dpi differed between those groups. The data indicate that CA-2-P100 virus caused a significantly limited viral load and a transient period of viremia compared to the parental CA-2-P20 virus. However, the virus that was additionally passaged in PAM cells (CA-2-MP120) regained viremia kinetics similar to those of the parental CA-2-P20 virus (up to 28 dpi) and in parallel with the overall reduced viral loads.

All pigs in Group 1 seroconverted by 10 dpi, and the mean sample:positive (S/P) ratio of the antibody peaked at 14 dpi, followed by a slight decline thereafter (panel B in Fig. 2). However, seroconversion occurred only in three pigs with viremia in Group 2, and their mean antibody titer continued to increase until the end of the experiment. The remaining three pigs failed to seroconvert to PRRSV until the termination of the study. Among these animals, one exhibited no viremia, another showed viremia at 35 dpi, and the other had intermittent viremia. Unlike the pigs in Group 2, all animals in Group 3 seroconverted by 10 dpi (5/6) or 14 dpi (1/6), and the highest peak of the mean S/P ratio was attained at 21 dpi and was sustained until the end of the study. The control pigs remained seronegative to PRRSV based on ELISA results. In addition, all virus-inoculated pigs were found to have absent ($< 1:2$) or low (1:4) neutralizing antibodies during the trial (data not shown). Overall, the data demonstrated that despite a delayed PRRSV-specific antibody response compared to that of the parental CA-2-P20 virus, CA-2-MP120 induced seroconversion in the natural host more frequently than CA-2-P100 did.
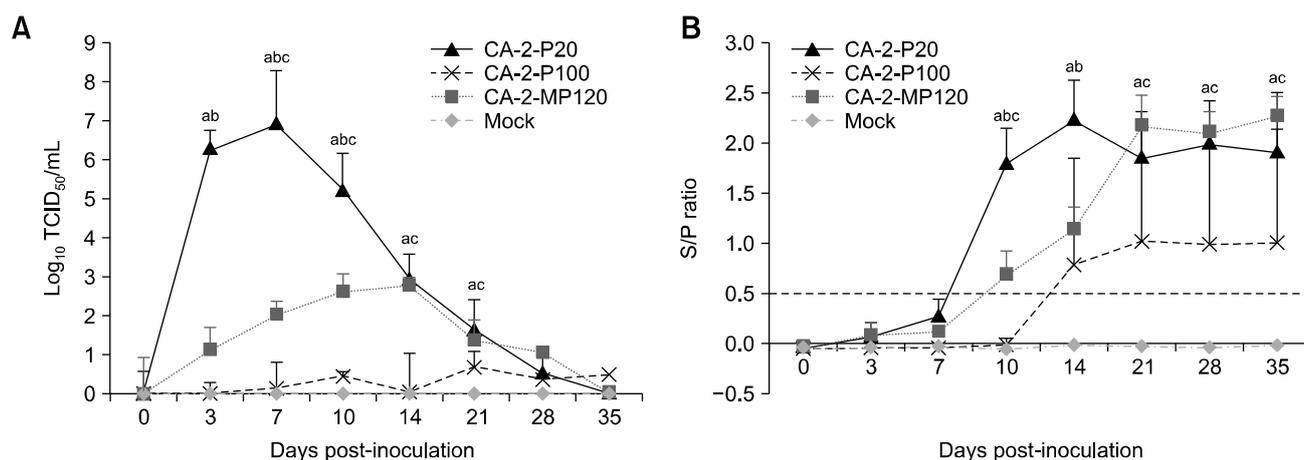

Fig. 2. Virus replication *in vivo* and seroconversion. (A) Mean levels of viremia of pigs infected with CA-2-P20, CA-2-P100, or CA-2-MP120. TCID$_{50}$, 50% tissue culture infectious dose. (B) Virus-specific antibody response of pigs measured by using a commercial enzyme-linked immunosorbent assay kit. Serum samples were considered positive for antibodies to porcine reproductive and respiratory syndrome virus if the sample:positive (S/P) ratio was equal to or greater than 0.4. Error bars represent SD. "a" denotes a significant difference ($p < 0.05$) between the CA-2-P20 and CA-2-P100 groups; "b" denotes a significant difference ($p < 0.05$) between the CA-2-P20 and CA-2-MP120 groups; "c" denotes a significant difference ($p < 0.05$) between the CA-2-P100 and CA-2-MP120 groups.

Gross pathological and histopathological changes

In general, macroscopic pathological lesions were comparable among the three PRRSV-infected groups and the control group. However, the lungs from all pigs in Group 1 had common microscopic features of acute PRRSV infection including interstitial pneumonia characterized by thickened alveolar walls, inflammatory cell infiltration, and perivascular and peribronchiolar cuffing. Inoculation with CA-2-P20 also caused minimal lymph node (LN) lesions with lymphoid follicular hyperplasia (Fig. 3). In contrast, only three viremic pigs in Group 2 had minimal interstitial pneumonia in the lung, while the remaining animals with no or sporadic viremia showed normal histopathological lung lesions analogous to those of the pigs in the control group. Furthermore, all pigs in Group 2 had no remarkable lesions in LNs. Similarly, four pigs in Group 3 displayed minimal lung lesions with either alveolar wall thickening or perivascular and peribronchiolar cuffing. Histopathological examination revealed minimal LN lesions in two animals in Group 3. The histopathologic lung scores were significantly higher in Group 1 than those in Groups 2 and 3 and were similar in the latter two cell-adapted virus-infected groups (Fig. 4). Therefore, our results indicated that CA-2-P100 exhibited a weakened virulence and, remarkably, the PAM-adapted CA-2-MP120 virus preserved this attenuated phenotype in the experimentally infected pigs.

Genome sequence analysis of PAM-passaged CA-2-P100-derivative strains

The sequence data analysis revealed no additional deletions or insertions in CA-2-MP110 and CA-2-MP120 compared with CA-2-P100, resulting in an identical genome size of 15,018 nt (189-nt 5′ UTR, 14,678-nt protein-coding regions, and 151-nt 3′ UTR), excluding the poly(A) tail, in all three strains. The genome sequences of CA-2-MP110 and CA-2-MP120 had high levels of similarity (99.5%−99.8%) with the parental CA-2-P20 (GenBank accession No. KU512797) and cell-adapted CA-2-P100 (GenBank accession No. KU512805) strains at the nt level. CA-2-MP110 and CA-2-MP120 possessed 68 and 74 nt mutations, respectively, compared with CA-2-P20, and these changes were dispersed randomly throughout the genome. Although the number of nt substitutions was higher than that observed in CA-2-P100, the number of aa changes was nearly
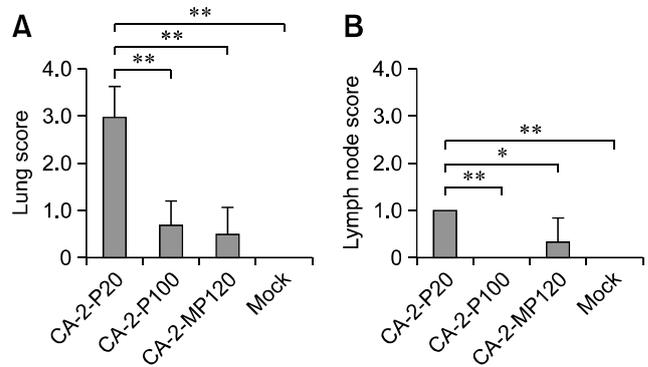

Fig. 4. Average histopathologic scores of lung (A) and lymph node (B) tissues from pigs in the CA-2-P20, CA-2-P100, CA-2-MP120, and control groups. The results are expressed as mean values from 6 pigs in each group, and error bars represent SD. $*p = 0.001-0.05$, $**p < 0.001$.
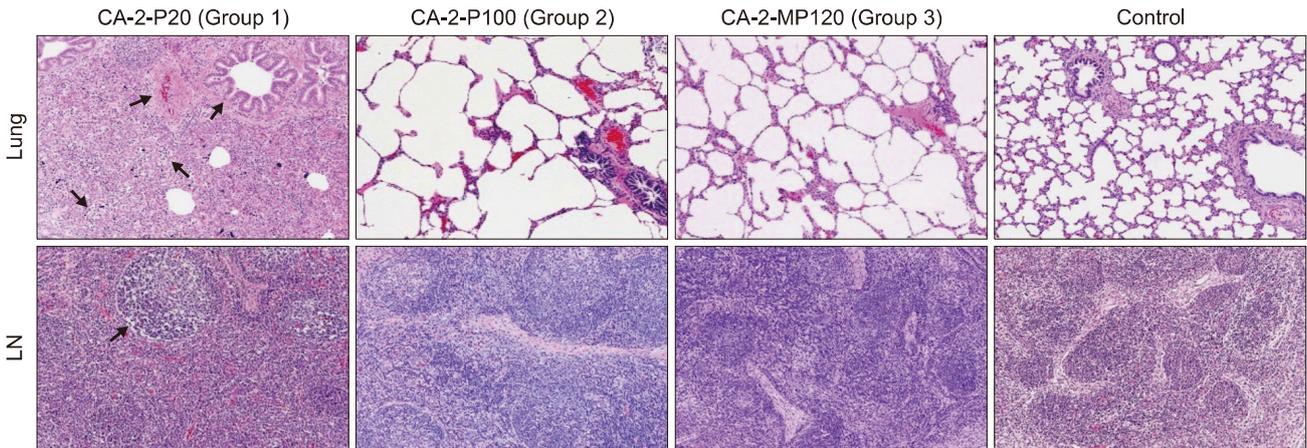

Fig. 3. Photomicrographs of the lungs and lymph nodes (LN) from pigs in the CA-2-P20, CA-2-P100, CA-2-MP120, and control groups. H&E-stained tissues from the pigs in each group were histopathologically examined. 100×. The upper panels represent lung sections from pigs of each group. A CA-2-P20-infected pig (Group 1) shows a marked interstitial pneumonia with alveolar walls thickened by massive lymphocyte and macrophage infiltration surrounding perivascular cuffing (arrows), whereas pigs inoculated with CA-2-P100 (Group 2) or CA-2-MP120 (Group 3) exhibit normal lung lesions comparable to control pig lung. The lower panels represent LN sections from pigs of each group. A pig in Group 1 displayed lymphoid follicular hyperplasia (arrow), whereas pigs in Groups 2 and 3 exhibited no remarkable LN lesions, as was observed in control pig LN samples.

identical to that in CA-2-P100, compared with CA-2-P20 (Fig. 5). Both ends of genomes of CA-2-MP110 and CA-2-MP120 contained equivalent 3 nt mutations, comprising one C-to-T substitution at position 59 in the 5′ UTR and two C-to-T substitutions at positions 30 and 144 in the 3′ UTR (positions 14,897 and 15,011, respectively, in the genome). Because those mutated nts are naturally found at corresponding positions in the other PRRSV-2 strains, they are unlikely to be related to *cis*-acting elements that have critical roles in genomic replication. These data indicated that neither the 5′ nor 3′ UTRs was involved in attenuation of the PRRSV.

The remaining 65 nt substitutions in CA-2-MP110 were in the coding regions, and 27 of these mutations were non-synonymous, causing aa changes. Similarly, the remaining 71 nt substitutions of CA-2-MP120 resulted in the same number of non-silent mutations as that in CA-2-MP110. There were seven different aa changes between CA-2-MP110 and CA-2-MP120. These included A680T, L872F, N948D, and A1255T in nsp2; G1350S in nsp12; L253Q in GP3; and G164K/R in GP5, which arose only in either CA-2-MP110 or CA-2-MP120. Among the 27 aa substitutions in CA-2-MP120, seven mutations newly emerged during passaging in PAM cells, whereas the remaining 18 mutations present in CA-2-P100. The remaining two variations occurred only in CA-2-P90 and re-emerged in CA-2-MP110. The mutations were distributed randomly in ORFs 1a, 1b, 2a, 2b, 3, 4, 5, and 6, which encode 13 viral proteins (7

nonstructural and 6 structural) (Table 1). Among the mutations, 51.9% (14/27) were dispersed in nsps, whereas 48.1% (13/27) were located in structural proteins. The proteins nsp1α, nsp2N, nsp3, nsp6, nsp7α, nsp7β, nsp8, nsp10, nsp11, nsp12, ORF5a, and N were highly conserved and remained unchanged during passaging in PAM cells. For confirmation of the NGS results, the genomic regions of CA-2-MP110 and CA-2-MP120 spanning the 27 aa substitutions were sequenced by using a traditional method. The mutated sequences of both viruses were identical to those identified by NGS.

When compared with CA-2-P100, the newly generated strains CA-2-MP110 and CA-2-MP120 contained 29 and 37 nt mutations, respectively, distributed randomly throughout the genome. As expected, the 5′ and 3′ UTRs remained virtually unchanged except for two C-to-T substitutions at position 59 in the 5′ UTR and position 144 in the 3′ UTR, which were identified in both CA-2-MP110 and CA-2-MP120. The remaining 27 and 35 nt substitutions in CA-2-MP110 and CA-2-MP120 were in the coding regions, leading to 14 and 15 aa changes, respectively (Fig. 5). Most mutations overlapped in the two PAM-passaged viruses, except for two mutations (L253Q in GP3 and R164K in GP5) and five mutations (A680T, L872F, D948N, and A1255T in nsp2 and G1350S in nsp12) identified only in CA-2-MP110 or CA-2-MP120, respectively. The 15 aa mutations in CA-2-MP120 were dispersed randomly in ORFs 1a, 1b, 2a, 2b, 3, 5a, and 5, which encode 9 viral
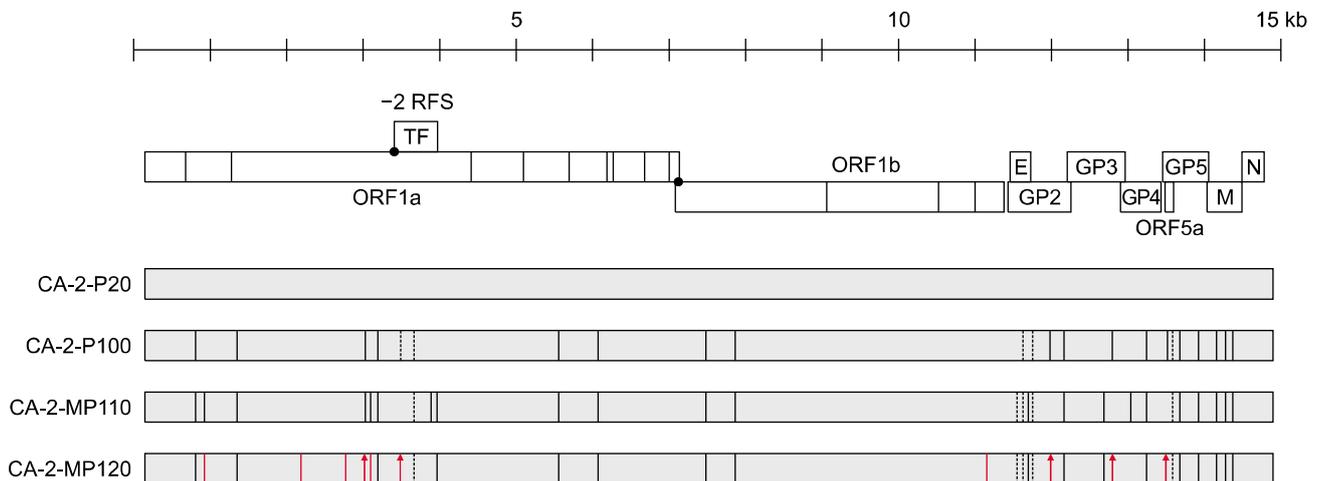

Fig. 5. Schematic representation of amino acid (aa) differences between the parental CA-2-P20 virus and its cell-adapted derivatives. The genomic sequences of the virus strains were determined as described in Table 1. The organization of the CA-2 genome (approximately 15 kb) is shown at the top. The coding regions for each nonstructural protein (nsp) in open reading frames (ORFs) 1a and 1b and for each structural protein are indicated. The lower panels show the genomes of the parental CA-2-P20 strain and its cell-adapted derivatives, where the vertical lines (black) represent a 1 aa substitution relative to the predicted aa sequence of CA-2-P20. The vertical dotted lines denote a 1 aa substitution in the overlapping coding regions for nsp2/nsp2TF, GP2/E, and GP5/ORF5a (silent mutation in nsp2, whereas a non-silent mutation in nsp2TF; silent mutation in GP2, whereas a non-silent mutation in E; non-silent mutation in GP5, whereas a silent mutation in ORF5a). The vertical lines (red) in the PAM-passaged CA-2-MP-120 strain indicate 15 aa changes relative to the predicted aa sequence of CA-2-P100 and the vertical arrows (red) indicate reverting mutations that restore the sequence of CA-2-P20.

Table 1. Amino acid (aa) mutations among CA-2-P20 and the derivative viruses upon cell adaptation

ORF	Encoding protein (aa length)	aa position*	Mutation at indicated No. of passages			
			CA-2-P20	CA-2-P100	CA-2-MP110	CA-2-MP120
ORF1a	nsp1β (203)	194	V	L	L	L
		220	L	L	F	**F**[†]
	nsp2 (1,065)	394	S	R	R	R
		680	A	A	A	**T**
		872	L	L	L	**F**
		948[‡]	N	D	D	**N**
		983	M	M	I	**I**
		985	L	P	P	P
		1255	A	A	T	A
		1291	S	S	A	**A**
	nsp2TF (888)	1108	I	T	I	**I**
		1146	L	P	P	P
	nsp4 (204)	1858	S	N	N	N
	nsp5 (170)	1981	T	I	I	I
ORF1b	nsp9 (685)	121	I	V	V	V
		238	R	C	C	C
	nsp12 (153)	1350	G	G	G	**S**
ORF2a	GP2 (256)	32	S	S	L	**L**
		91	I	F	I	**I**
		166	L	M	M	M
ORF2b	E (73)	3	T	T	A	**A**
		9	D	Y	Y	Y
		48	L	F	F	F
ORF3	GP3 (254)	137	A	A	V	**V**
		162	S	P	S	**S**
		253	L	L	Q	L
ORF4	GP4 (178)	141	F	S	S	S
ORF5a	5a (46)	12	G	E	G	**G**
ORF5	GP5 (200)	9	G	S	G	**G**
		34	N	S	S	S
		59	R	H	H	H
		164	G	R	K	R
ORF6	M (174)	63	T	A	A	A
		96	I	T	T	T
		107	K	R	R	R

ORF, open reading frame. *aa position numbering is based on the sequence of the CA-2 strain. [†]aa in CA-2-MP120 that differ from CA-2-P100 are shown in boldface type. [‡]Reverting mutations in CA-2-MP120 that are identical in CA-2-P20 are shaded.

proteins (4 nonstructural and 5 structural) (Table 1). Among these mutations, 53.3% (8/15) were in nsps, whereas 46.7% (7/15) were in structural proteins. The proteins nsp1α, nsp1β, nsp2N, nsp3−6, nsp7α, nsp7β, and nsp8−11, GP4, M, and N contained no mutations. Among the 15 mutations, seven newly arose during passaging in PAM cells, whereas the remaining eight were reversions to the aa sequences of the previously reported MARC-145 cell-passage derivatives of CA-2. The mutated sequences of CA-2-MP120 were further confirmed by

sequencing the regions covering the aa substitutions. Taken together, the results suggested that the molecular determinants of the macrophage tropism of PRRSV are equally distributed between nonstructural and structural proteins.

Amino acid mutations of PAM-passaged CA-2-P100-derivative strains

ORFs 1a and 1b of CA-2 encode 2,372- and 1,458-aa polyproteins, respectively, which are predicted to be

post-translationally cleaved into 16 nsps. Eight aa changes in CA-2-MP120 were distributed among four functional nsps including nsp1β, nsp2, nsp2TF, and nsp12, which individually contained one, five, one, and one mutation(s), respectively, in comparison to CA-2-P100 (Table 1). Most (62.5%) of the mutations (5/8) accumulated in nsp2, which is the most variable region within the PRRSV genome, and four of those mutations (A680T, L872F, D948N, and M983I) were concentrated in its HV2 region. The first two variations were newly identified in CA-2-MP120, while the remaining mutated residues resulted from reversion to the parental strain sequence or the strain generated at the 90th passage in MARC-145 cells [22]. Since two novel products generated by −2 RFS within nsp2 (nsp2TF and nsp2N) are truncated nsp2 variants sharing the N-terminal two-thirds region of nsp2, those four aa mutations were identically located in the two truncated nsp2 proteins. The last mutation (S1291A) was in the C-terminus of nsp2 and emerged in CA-2-MP120. Intriguingly, two changes (D948N in nsp2 and T1108I in nsp2TF) restored the sequence of the parental strain CA-2-P20.

In comparison to CA-2-P100, seven aa mutations were found in ORF2, ORF3, and ORF5, which encode five structural proteins (GP2, E, GP3, ORF5a, and GP5) of CA-2-MP120 (Table 1). Among those, four mutated aa residues restored the sequence of the parental CA-2-P20 strain, while two mutations in GP2 (S32L) and E (T3A) emerged only in CA-2-MP120. Unexpectedly, GP5 had one substitution (S9G), while aa residues involved in two epitopes and three potential N-glycosylation sites within GP5 remained unchanged throughout the PAM cell passages. Interestingly, 4/7 variations in the structural protein-coding region of CA-2-MP120 were in minor structural proteins, GP2 (S32L and F92I) and GP3 (A137V and P162S). In particular, four changes (F92I in GP2, P162S in GP3, and E12G/S9G in ORF5a/GP5) in CA-2-MP120 restored the sequence of the parental CA-2-P20 strain. No mutations arose in the GP4, M, and N protein-coding regions.

Discussion

Previously, we applied a traditional method for viral attenuation by passaging the virulent nsp2 DEL strain CA-2 in MARC-145 cells 100 times. However, the obtained derivative strain, CA-2-P100, was incompletely capable of inducing viremia and seroconversion in pigs under experimental conditions, suggesting potential shortcomings as a vaccine candidate. These *in vivo* outcomes might have resulted from the possibility that CA-2-P100 lost its macrophage-tropic phenotype after it was serially cultivated in MARC-145 cells, which are from neither pig nor the monocyte-macrophage lineage. In this study, we sought to obtain a macrophage-tropic CA-2-MP120 strain by passaging CA-2-P100 in PAM cells 20 times to develop a possible PRRSV vaccine candidate and

investigated its pathogenic and genetic traits derived from the macrophage adaptation process.

CA-2-P20 and its cell-passaged derivatives, CA-2-P100 and CA-2-MP120, were characterized in pigs and their pathogenicities were compared. We were unable to observe altered clinical symptoms in all pigs inoculated with CA-2-P100 and CA-2-MP120 compared with those of non-infected control pigs. However, the febrile response was distinguishable between the parental and cell-adapted virus-infected groups. Consistent with the observations in our previous study [22], half of the pigs in the CA-2-P100 group had either no viremia or 1-day-viremia and were seronegative throughout the experiment. Therefore, both previous and current results suggest that CA-2-P100 virus, which was generated by extensive passaging in MARC-145 cells, might be over-attenuated. In contrast, all pigs inoculated with CA-2-MP120 exhibited viremia and robust antibody responses. Nevertheless, lung lesions in the CA-2-P100 and CA-2-MP120 groups were macroscopically and microscopically similar to those in the control group. Our experimental results suggested that CA-2-MP120 retained attenuated virological and clinical phenotypes in the natural host and could be considered as a vaccine candidate.

The functional nsps of PRRSV, namely nsp1α and nsp1β, are major antagonists of the host innate immune defense and are associated with PRRSV pathogenesis [3,29,30]. Thus, the single L220F mutation detected in nsp1β of both CA-2-MP110 and CA-2-MP120 may contribute to viral attenuation. PRRSV nsp2 is a large multifunctional gene with a variable nature, resulting in sequence heterogeneity and different genome lengths among PRRSV isolates. Accordingly, five out of 15 aa mutations appeared in nsp2 during the passaging of CA-2-P100 in PAM cells, and four of those mutations were clustered in the HV2 region of nsp2. Some specific regions of the nsp2 protein have been suggested as being highly associated with the cell and tissue tropism of PRRSV [9,31]. Therefore, we hypothesized that variation(s) in nsp2 could have contributed to the macrophage tropism of the PRRSV strains in this study and further influenced their replication and virulence *in vivo*. One mutation (D948N) in nsp2 that arose during passaging in PAM cells represented a reversion to the parental virus sequence, implying its importance for the cell tropism of PRRSV. Moreover, another aa substitution (T1108I) that was identified throughout the serial passages in PAM cells, and which was located within the C-terminal domain of the −2 RFS-derived product nsp2TF, was also equal to that in the sequence of CA-2-P20, suggesting the potential for this novel protein to be associated with macrophage tropism.

Nearly half of the aa variations in CA-2-MP120 were in structural gene coding regions. The genetic data were consistent with those reported in our previous study [22], supporting the suggestion that nonstructural and structural proteins might

contribute in parallel to the virulence of the CA-2 strain. Considering the size of those regions, the mutations could have altered the virological and clinical features of CA-2-MP120. Although specific mutations such as E83G in GP3 and R151G in GP5 have been associated with viral virulence [2,5,20,33], we were unable to confirm those particular changes in the present study. Interestingly, we found four reverting mutations in CA-2-MP120 that restored the sequence of the reference CA-2 and parental CA-2-P20 strains. These reverting mutations included F92I in GP2, P162S in GP3, and E12G/S9G in ORF5a/GP5, which may be relevant to virulence in pigs. Meanwhile, two minor structural proteins, GP2 and GP3, each had two aa mutations in the PAM-passaged CA-2-MP120 virus. PRRSV GP2, GP3, and GP4 form a disulfide-linkage heterotrimeric complex in the viral envelope that is indispensable for infectivity [7,32]. Furthermore, CD163, the cellular receptor for PRRSV, interacts with GP2 and GP4 [7]. Therefore, the S32L and F92I mutations in GP2 that occurred during the serial passages in PAM cells may affect the cell tropism of PRRSV by facilitating the efficient association of the GP2-GP3-GP4 complex with porcine CD163, thereby contributing to the virulence of CA-2-MP120.

In conclusion, we found that the attenuated phenotype of the PRRSV cell-adapted nsp2 DEL strain CA-2-P100 was sustained throughout serial passages in PAM cells. Furthermore, the PAM-passaged CA-2-MP120 replicated more efficiently in pigs than CA-2-P100 did, suggesting that the virus became macrophage-tropic after PAM passages. Although a growing body of evidence proposes that virulence determinants could be strain-specific and polygenic, or could include cellular components, the genetic drift that arose during the PAM cell adaptation process might influence cell tropism and contribute to virulence-related parameters of CA-2-MP120. The reverting mutations of nsp2 and minor structural protein-coding regions identified in the present study may be important for macrophage tropism and pathogenicity, a suggestion that should be addressed by using a reverse genetics approach. The results of this study provide insights that may be pertinent during the development of a next-generation vaccine using the attenuated CA-2-MP120 virus. Experiments are in progress for assessing the potential of the attenuated CA-2-MP120 virus to revert and become virulent in order to address safety concerns and for evaluating the protective efficacy of this strain against homologous or heterologous PRRSV strains.

Acknowledgments

Conflict of Interest

The authors declare no conflicts of interest.

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