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Generation of porcine reproductive and respiratory syndrome virus by *in vitro* assembly of viral genomic cDNA fragments



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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent for a swine disease affecting the pig industry worldwide. Infection with PRRSV leads to reproductive complications, respiratory illness, and weak immunity to secondary infections. To better control PRRSV infection, novel approaches for generating control measures are critically needed. Here, *in vitro* Gibson assembly (GA) of viral genomic cDNA fragments was tested for its use as a quick and simple method to recover infectious PRRSV in cell culture. GA involves the activities of T5-exonuclease, Phusion polymerase, and Taq ligase to join overlapping cDNA fragments in an isothermal condition. Four overlapping cDNA fragments covering the entire PRRSV genome and one vector fragment were used to create a plasmid capable of expressing the PRRSV genome. The assembled product was used to transfect a co-culture of 293T and MARC-145 cells. Supernatants from the transfected cells were then passaged onto MARC-145 cells to rescue infectious virus particles. Verification and characterization of the recovered virus confirmed that the GA protocol generated infectious PRRSV that had similar characteristics to the parental virus. This approach was then tested for the generation of a chimeric virus. By replacing one of the four genomic fragments with that of another virus strain, a chimeric virus was successfully recovered *via* GA. In conclusion, this study describes for the first time the use of GA as a simple, yet powerful tool for generating infectious PRRSV needed for studying PRRSV biology and developing novel vaccines.

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an arterivirus that causes significant losses to the swine industry, making it one of the most economically important animal pathogens (Yun and Lee, 2013). PRRSV belongs to the *Arteriviridae* family within the *Nidovirales* order. Its genome is composed of a positive-sense, single-stranded RNA with an approximate size of 15 kb. Pigs infected with PRRSV display symptoms related to the reproductive and respiratory systems. Abortion and premature farrowing are often observed in infected sows, while pneumonia and increased susceptibility to secondary infections can be observed in infected pigs of all ages (Rossow, 1998). Current strategies for controlling PRRSV outbreaks include the use of modified

live-attenuated vaccines (MLVs) and inactivated vaccines (Kimman et al., 2009). However, these immunization strategies are not fully protective against PRRSV infection. Current MLVs have been associated with numerous problems such as shedding of vaccine virus, persistent infection, incomplete protection and reversion to virulence, while the reported outcomes for inactivated vaccine use have not been promising (Huang and Meng, 2010).

Understanding the molecular basis of PRRSV virulence is critical for developing better PRRSV vaccines. A reverse genetics system could provide a powerful tool for dissecting the role of viral proteins in the viral life cycle and pathogenicity. One of the most definitive ways to study the roles of specific sequences in a viral genome is to modify them and use them to generate infectious virus—that is, to ‘rescue’ virus with these modified sequences (Bridgen, 2012). Various approaches have been employed to generate PRRSV infectious clone, including the use of a bacterial artificial chromosome (Wang et al., 2013), a T7 promoter in the low copy-number plasmid pOK12 (Meulenberg et al., 1998; Nielsen et al., 2003), a CMV promoter-driven plasmid (Lee et al., 2005), and an RNA-launched system

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Table 1
List of primers for the generation of cDNA fragments and the linear vector.

Primers	Genomic positions	Sequences (5'–3')
F1F	1–20	GCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTGTGAAACCGTTATGACGTATAGGTGTTGGC
F1R	3442–3491	CGAGAAAGCCATTCTCGGTAGCAGGGTATCAAGCTTAGTCGCATCAC
F2F	3491–3442	GTGATGCGACTAAGCTGTATGACCCGTGCTACGCAGGAATGGCTTTCTCG
F2R	7131–7082	GATAAATTGACCCGCTGCATTCCTCATGTGGACGCAGAAACAAAGCAC
F3F	7082–7131	GTGCTTTGTTTCTGCGTCCAACATGAGGAATGCAGCGGGTCAATTTATC
F3R	11154–11202	GTAGATTCTCTCCGCGCCATTTGTGCTGATCTAGAAGGGTCGAGCTCTC
F4F	11154–11202	GAGAGCTCGACCTTCTAGATCAGCACAAATGGCGCGGAGAGAATCTAC
F4R	15393–15412	GCGAGAGGTTGGAGATGCCATGCCGACTTTTTTTTTTTTTTTTTTTTAATTCGGCCGCATGGTTCT
Vector-F	Poly(A) tail	AAAAAAAAAAAAAAAAAAAAAAAAAGTCGGC
Vector-R	none	ACGGTTCCTAAACGAGCTC

(Fang et al., 2006). All of these bacterial-clone approaches require substantial effort, are time consuming, and are prone to introducing undesired mutations during amplification of plasmid DNA in bacteria. One alternative approach for generating an infectious PRRSV clone is to use an *in vitro* assembly method, such as the Gibson assembly (GA) method, originally described by Gibson et al. in 2009 (Gibson et al., 2009). This method involves three kinds of enzymes: T5 exonuclease, a DNA polymerase and a DNA ligase, which act in a single-tube reaction to assemble multiple overlapping DNA molecules (Gibson et al., 2009). The T5 exonuclease functions in the assembly process by chewing 5' ends of the overlapping DNA fragments. The digested DNA fragments can then be seamlessly joined together by the DNA polymerase, which adds the complementary nucleotides, and the DNA ligase, which fills in the gaps between the DNA fragments. *In vitro* DNA assembly methods, such as GA, have been used successfully to assemble a complete synthetic *Mycoplasma genitalium* genome (Gibson et al., 2009), mouse mitochondrial genome (Gibson et al., 2010), and dengue virus genome (Siridechadilok et al., 2013). To our best knowledge, there has been no previous report on the use of GA to generate PRRSV. This study describes a protocol for generating PRRSV directly from an *in vitro* GA reaction. Genome sequencing analysis and characterization of the recovered virus suggest that GA could serve as an efficient platform to generate infectious PRRSV. It also shows that GA can be used as a simple tool to generate chimeric viruses. By swapping one of the genomic fragments of one virus with that of another virus, a chimeric virus was successfully generated. GA is thus a simple yet powerful tool for generating genetically modified PRRSV.

2. Materials and methods

2.1. Cells, viruses, and plasmids

293T cells and MARC-145 cells were maintained in Opti-MEM (Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS). All cells were maintained at 37 °C, at 5% CO₂ and 80% humidity. The viruses used in this study were from commercial vaccines: the modified live-attenuated North American RespPRRSV from Ingelvac PRRS MLV (Boehringer, Germany, Genbank accession number AF066183 (Opriessnig et al., 2002)), and a live attenuated HP-PRRSV Hun4 F112 strain (Harbin Veterinary Research Institute, China (Tian et al., 2009)). Both viruses were cultured in MARC-145 cells for several passages to obtain high titer stocks. The pSMART BAC expression vector (Lucigen, USA) was modified based on reported modifications designed to generate a coronavirus infectious clone (Almazan et al., 2000, 2013). Modifications included the addition of a CMV promoter, a stretch of 25 adenosine residues (A₂₅), the hepatitis delta virus (HDV) ribozyme, and the bovine growth hormone (BGH) poly(A) signal. Such modifications were shown to allow efficient generation of positive-sense, capped and polyadenylated viral RNA (vRNA) (Almazan et al., 2000,

2013). pGFP used as transfection control was generated in our laboratory by inserting green fluorescence protein (GFP) gene into the modified pSMART BAC. pJet1.2 was purchased from Thermo Scientific (USA).

2.2. RNA isolation and RT-PCR

vRNA was extracted from 200 µl of cell culture supernatant of MARC-145 cells infected with RespPRRSV for 3 days using the Viral Nucleic Acid Extraction Kit II according to the manufacturer's protocol (Geneaid, Taiwan). Viral cDNA synthesis was performed with the RevertAid Premium first-strand cDNA synthesis kit according to the manufacturer's protocol (Thermo Scientific). Primers for cDNA synthesis were designed to bind the center of the genome (F2R primer) and the 3' end of the genome (F4R primer) (see Table 1 for primer sequences). PCR products for RespPRRSV genome assembly were generated using high-fidelity DNA polymerase KAPA HiFi (KAPA Bioscience, USA) according to the manufacturer's protocol. A set of primers was designed to generate 4 PCR fragments (F1, F2, F3, and F4) covering the entire genome, each fragment staggered to generate 49-bp overlaps with the neighboring fragments. The forward primer of F1 and the reverse primer of F4 were designed to have overlapping nucleotides with the modified pSMART BAC vector. Similarly, the forward primer for amplifying the vector was designed to have overlapping nucleotides with the 3' end of F4. The melting temperatures of all primers were between 52 and 65 °C. Primer sequences are listed in Table 1. All PCR products were subjected to gel electrophoresis, and then cleaned up with the GenepHlow Gel/PCR Kit (Geneaid) according to the manufacturer's protocol. The PCR product of vector was also treated with DpnI to digest the original circular plasmid template.

2.3. GA reactions

The full-length RespPRRSV genome was assembled *in vitro* as illustrated in Fig. 1. Briefly, after gel clean-up, PCR products of both the modified pSMART BAC vector and the four viral cDNA fragments were assembled together in an isothermal GA reaction (Gibson et al., 2009). Fragments were combined with a home-made 1.3 × GA master mix, prepared as previously described (Gibson et al., 2009). Briefly, 699.36 µl water, 320 µl 5 × isothermal reaction buffer (25% PEG-8000, 500 mM Tris-Cl, pH 7.5, 50 mM MgCl₂, 50 mM DTT, 1 mM of each of the four dNTPs, 5 mM NAD), 0.64 µl T5 exonuclease (10 U/µl, Epicentre, USA), 20 µl Phusion DNA polymerase (2 U/µl, Thermo Scientific) and 160 µl Taq DNA ligase (40 U/µl, New England Biolabs, USA) were combined. The resulting solution was divided into 15 µl aliquots and stored at –20 °C.

In the assembly process, 100 ng of DpnI-treated linear modified pSMART BAC vector was added to 15 µl of 1.3 × master mix containing equal amounts of the RespPRRSV-derived PCR products (within the range of 100–110 ng) for a total assembly reaction volume of

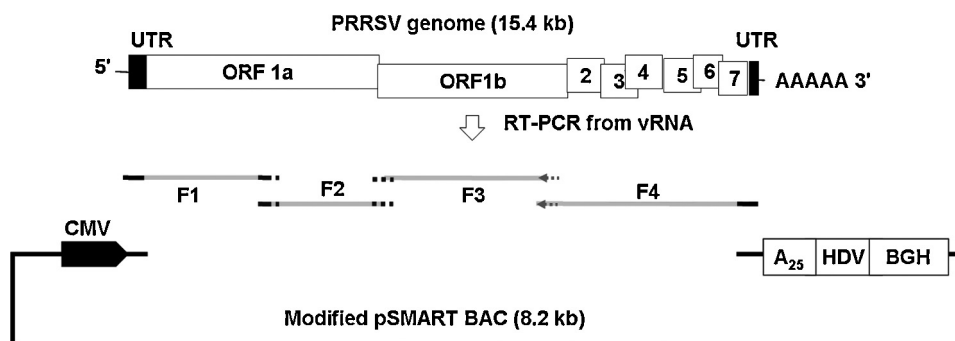


Fig. 1. Design of GA construct for generating PRRSV in cell culture. Four overlapping sets of primers were used to generate 4 pieces of cDNA covering the entire genome of PRRSV. All cDNA fragments were assembled into the modified pSMART BAC which contained overlapping nucleotides with F1 and F4 fragments. While the CMV promoter functions as the recognition site for host RNA polymerase II to transcribe positive-sense, capped genomic RNA, the A₂₅ and the HDV ribozyme guarantee that the transcripts contain poly(A) tails of sufficient length for stability.

20 μ l. The assembly reaction was performed at 50 °C for 1 h and kept on ice before further experiments. To generate the chimeric virus, an F4 fragment encoding ORFs 2–7 of the Hun4 F1 12 strain was generated using the above protocol and mixed with F1–F3 fragments from RespPRRSV.

2.4. Virus recovery

The 20 μ l GA reaction mixture was diluted in 80 μ l serum-free Opti-MEM medium and mixed with 3 μ l Metafectene-Pro (Biontech, Germany) according to the protocol provided by the manufacturer. The mixture was transfected into a co-culture of 293T cells (at 60% confluence or 6×10^5 cells/well) and MARC-145 cells (at 40% confluence or 2×10^5 cells/well) in 6-well plates. The culture supernatant was harvested on day 3 post-transfection and adsorbed at 37 °C on MARC-145 cells that were grown in a six-well plate overnight. After 1 h, the supernatant was removed, and the cells were washed 3 times with $1 \times$ PBS and subsequently replenished with Opti-MEM containing 10% heat-inactivated FBS. Recovered viruses were harvested along with the supernatants, and then stored at –80 °C for subsequent infection, virus titration, and viral RNA extraction.

2.5. Virus genome sequencing

On day 3 after the second viral passage in MARC-145 cells, infectious supernatants were collected for vRNA extraction. DNA sequencing of the viral genome was performed on RT-PCR products derived from the vRNA. The list of sequencing primers is available upon request.

2.6. Comparison of virus characteristics

The recovered GA-derived virus was compared against the parental RespPRRS virus in terms of plaque size, CPE, and growth kinetics. To perform the plaque assay, serial dilutions (10-fold) of the harvested culture medium were used to infect MARC-145 cells grown in 6-well culture plates. Plates were incubated at 37 °C for 1 h, with gentle rocking every 15 min. Plates were then washed twice with $1 \times$ PBS following removal of the culture medium. Medium containing 0.9% agar was used to overlay the culture. After 4 days of incubation, 0.1% crystal violet solution was used to visualize plaque formation.

To study growth kinetics, MARC-145 cells were infected in 24 well-plates at multiplicity of infection (MOI) of 0.01. After virus adsorption for 1 h at 37 °C, the cells were washed three times with $1 \times$ PBS, then supplemented with fresh Opti-MEM containing 10% heat-inactivated FBS. Supernatants were collected for virus

titration at 0, 12, 24, 48 and 72 h post-infection. CPE was noted during the growth curve assay.

2.7. Restriction fragment length polymorphism

RT-PCR reactions using primers specific to the 5' end (F1 region) and the 3' end (F4 region) of the viral genome were performed. The PCR products were purified and digested with restriction enzymes AatII and NaeI for the 5' end products and with EcoRV and NruI for the 3' end products. Digested products were analyzed on a 1% agarose gel by ethidium bromide staining.

3. Results

3.1. Design of the GA-derived construct

To assemble the RespPRRSV genome *in vitro*, four cDNA fragments covering the entire RespPRRSV genome were designed to be inserted into a modified pSMART BAC plasmid, which contains a CMV promoter, a 25-nucleotide poly(A) stretch (A₂₅), the hepatitis delta virus (HDV) ribozyme sequence, and the bovine growth hormone (BGH) poly(A) signal (Fig. 1). The cDNA fragments, named F1, F2, F3, and F4, were designed to cover the entire genome of RespPRRSV. Their lengths were limited to 3–5 kb to minimize the number of cDNA fragments and to avoid difficulties in amplifying extremely large cDNA fragments. To facilitate assembly, all fragments contained approximately 49 bases that overlapped with the ends of flanking cDNA fragments or the linear vector. The linear vector itself was obtained by PCR amplification of the modified pSMART BAC, starting from the A₂₅ to the CMV, in the 5'–3' direction. Upon assembly, the cDNA fragments were expected to be precisely inserted between the CMV and the A₂₅ sequences. The CMV promoter would be located at the 5' end of the genome, and function as the recognition site for host RNA polymerase II to transcribe positive-sense, capped genomic RNA. The A₂₅ and the HDV ribozyme sequences located at the 3' end of the viral genome would guarantee that the transcripts contain poly(A) tails of sufficient length for stability. The expected size of the assembled product is 23.6 kb.

3.2. GA reactions

The materials needed for the generation of RespPRRSV by GA include four cDNA fragments and the linear vector, which were prepared as follows. First, RT-PCR of purified RespPRRSV vRNAs was performed. Only 3 fragments, F1–F3, were successfully generated directly from the purified vRNA. The RT-PCR yields of F4 were insufficient for downstream applications. The product was

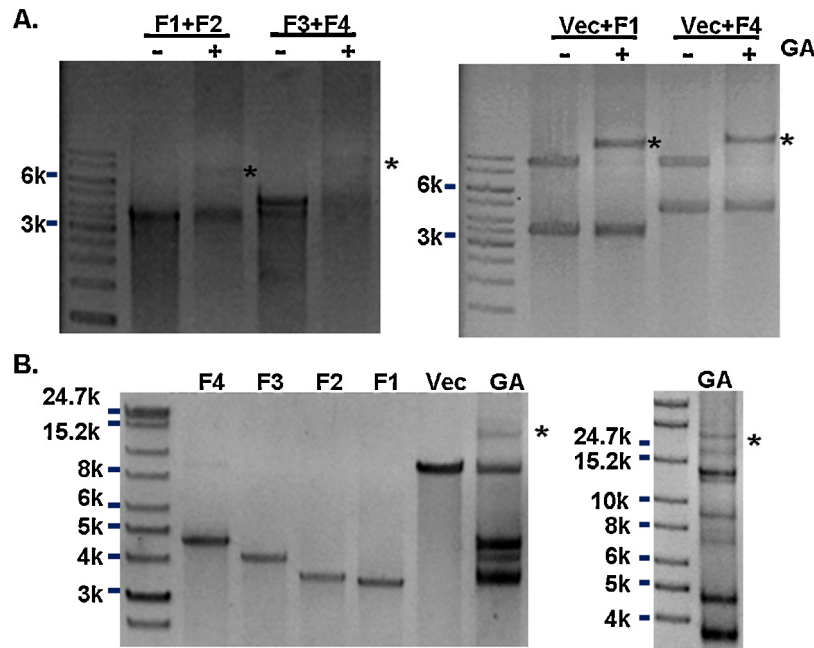


Fig. 2. GA reactions. (A) F1 and F2 (lane 1), or F3 and F4 (lane 3) (left panel), or F1 and vector (lane 1), or F4 and vector (lane 3) (right panel) were added to the GA reaction mixtures and incubated at 50 °C for 1 h. GA products are shown in lanes 2 and 4 of each panel, respectively. Asterisks indicate expected larger sizes of GA-derived DNA bands. (B, left panel) Lanes 1–5 show the sizes of purified F4, F3, F2, F1, and the linear vector, respectively. Lane 6 shows the GA products obtained from a reaction containing all four cDNA fragments and the linear vector. The asterisk indicates a large DNA band at approximately 23 kb. All DNA fragments were examined on a 1% agarose gel (left panel). GA products were analyzed on a 0.5% agarose gel for better separation of the DNA bands (right panel). The asterisks indicate a large DNA species of around 23 kb.

therefore cloned into the pJet1.2 cloning vector to be used as a template for generating the F4 PCR product. The modified pSMART BAC expression vector was PCR amplified to generate linear dsDNA with nucleotides overlapping with the RespPRRSV genomic sequence.

All cDNA fragments were purified and tested for their ability to assemble with their neighboring cDNA fragments. Assembling reactions were carried out in a home-made GA master mix prepared as described in Section 2 (Gibson et al., 2009). As indicated by the asterisks in the left panel of Fig. 2A, DNA products of larger sizes were generated after F1 and F2 were added to the home-made master mix for 1 h at 50 °C. Similar results were also observed when F3 and F4 were used, suggesting that the cDNA fragments contained appropriate overlapping nucleotides to facilitate fragment assembly. Next, assembly of the cDNAs with the linear vector was examined. F1, which overlaps with the 3' end (CMV region) of the linear vector, or F4, which overlaps with the 5' end (A_{25} region) of the linear vector, was incubated with the linear vector in the home-made master mix. As shown in the right panel of Fig. 2A, both F1 and F4 fragments were able to connect to the modified pSMART BAC vector, as indicated by detection of a larger DNA species among the GA-derived products (marked with asterisks). Given successful assembly of the cDNA fragments to each other and to the vector, all four viral cDNA fragments were then added to the master mix together with the modified pSMART BAC vector. Larger bands at the approximate size of 23 kb were documented on agarose gels following the GA reaction (Fig. 2B), suggesting that the cDNA fragments were successfully inserted into the pSMART BAC vector.

3.3. Recovery of GA-derived PRRSV

To recover infectious PRRSV from the GA-derived products, a co-culture of 293T cells and MARC-145 cells was transfected with the GA mixture. 293T cells were included in the coculture because they can be efficiently transfected, while MARC-145 cells were chosen due to their permissiveness to PRRSV infection. Five days after transfection, CPE was observed in cells transfected with the GA

mixture. CPE was also observed in cells transfected with purified PRRSV vRNA, but no CPE was apparent in the control cells transfected with the pGFP control plasmid (data not shown). To verify the presence of infectious virus particles in the transfected cultures, supernatants of the transfected co-cultures were passaged onto MARC-145 cells for CPE observation. Cells exposed to the GA-transfection supernatant started to show CPE on day 3, similar to cells exposed to the vRNA-transfection supernatant (Fig. 3A). No CPE was noted in cells exposed to the pGFP-transfection supernatant (Fig. 3A). To confirm that GA-derived PRRSV was generated, a two-step RT-PCR was conducted using specific primers for PRRSV ORF7. RNA extracted from the supernatant of cells exposed to the GA mixture was used as a template to perform two-step RT-PCR. As shown in Fig. 3B, a strong band at the expected size of 370 bp was observed, suggesting the presence of vRNA in the culture supernatant. The amplification was specific to the vRNA and not the potential residual cDNA fragments, as suggested by the absence of any amplified product in the reaction lacking reverse transcriptase (–RT, Fig. 3B). Together, these data suggest that PRRSV particles containing vRNA were released into the supernatant of the GA-treated culture.

3.4. Characterization of the GA-derived virus

To examine whether the virus recovered from the GA-treated culture resembled the RespPRRSV virus, genomic sequences of the recovered virus were analyzed. Out of 15,412 bases of the viral genome, nine nucleotides of the GA-derived virus were found to be different from the RespPRRSV. While the majority of these nucleotide changes were synonymous mutations, three of them were non-synonymous. A close examination of the sequence chromatograms of the original RespPRRSV virus revealed that the nucleotides leading to non-synonymous amino acid changes, including C3177T, G3435T, and G5322A, all displayed overlapping peaks, suggesting the co-existence of two nucleotide species (Fig. 4A). Although the major peaks were considered to be canonical

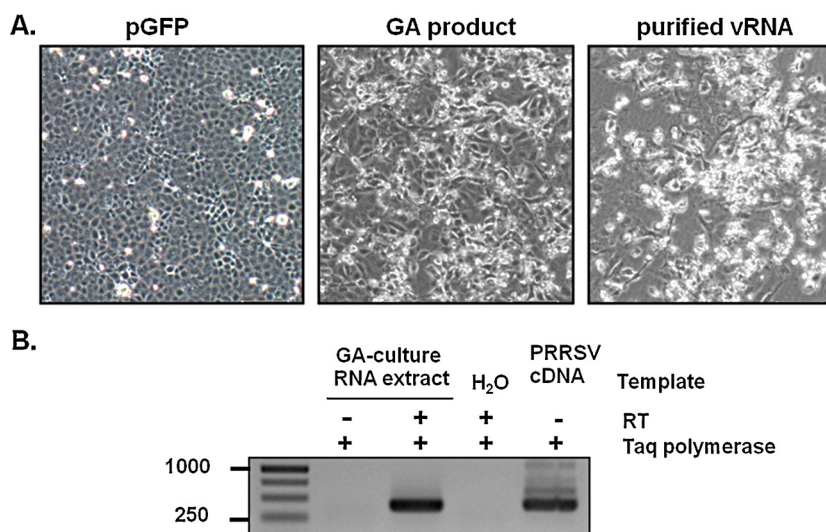


Fig. 3. Verification of GA-derived virus. (A) Co-cultures of 293T and MARC-145 cells were transfected with 1 μ g of pGFP, GA product (with all 4 cDNA fragments and the linear vector), or purified RespPRRSV vRNAs. Supernatants of the transfected co-cultures were used to infect MARC-145 and CPE was documented on day 3. (B) RNA was isolated from the supernatants of MARC-145 cells exposed to co-culture supernatants. Two-step RT-PCR using primers specific for PRRSV ORF7 was performed with or without reverse transcriptase (RT). Pre-made cDNA of RespPRRSV was used as a positive control for the PCR reaction.

sequences, the minor peaks found in the RespPRRSV genome became the major population of the GA-derived virus, suggesting that the parental RespPRRSV stock may already contain a minor population with these three non-synonymous mutations. Further sequence analysis revealed that the C3177T mutation was also present in the virus recovered from the transfection of the vRNA, but the nucleotides at positions 3435 and 5322 were guanine residues similar to the parental virus. This suggests that the C3177T mutation may be induced by an adaptation to the 293T-MARC-145 coculture or serial passages in MARC-145 cells,

while the G3435T and G5322A were specific mutations amplified during the GA process. To examine whether growth characteristics of the GA-derived virus resembled that of the parental virus, growth curve experiments were performed. As shown in Fig. 4B, the rescued GA-derived virus exhibited a growth rate similar to that of the RespPRRSV. Moreover, CPE patterns and plaque sizes were similar in cells infected with either virus (Fig. 4C and D). Together, these results suggest that GA could be used to generate PRRSV *in vitro* to yield virus with parental growth characteristics.

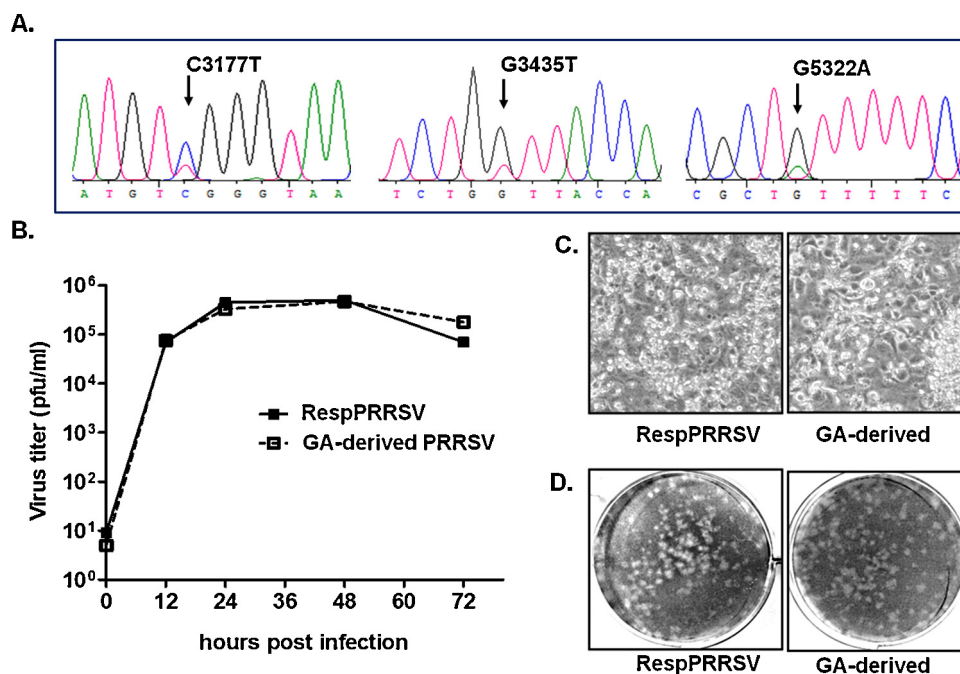


Fig. 4. Characterization of the GA-derived PRRSV. (A) Chromatograms of the original RespPRRSV virus display the nucleotide positions found to cause non-synonymous mutations in the GA-derived virus. Overlapping peaks representing two nucleotide species are indicated by the arrows. Numbers indicate the nucleotide position from the 5' end of the genome. (B) MARC-145 cells were infected at an MOI of 0.01 with RespPRRSV or GA-derived PRRSV. Supernatants of the infected cultures were collected at 0, 12, 24, 48, and 72 h post-infection, and titrated via plaque assay. (C) MARC-145 cells were infected at an MOI of 0.01 with RespPRRSV or GA-derived PRRSV. CPE was documented on day 3 post-infection. (D) Plaque assays were performed on MARC-145 cells infected with the RespPRRSV or GA-derived PRRSV. On day 4 post-infection, the overlaid medium containing 0.9% agar was removed and cells were stained with crystal violet solution.

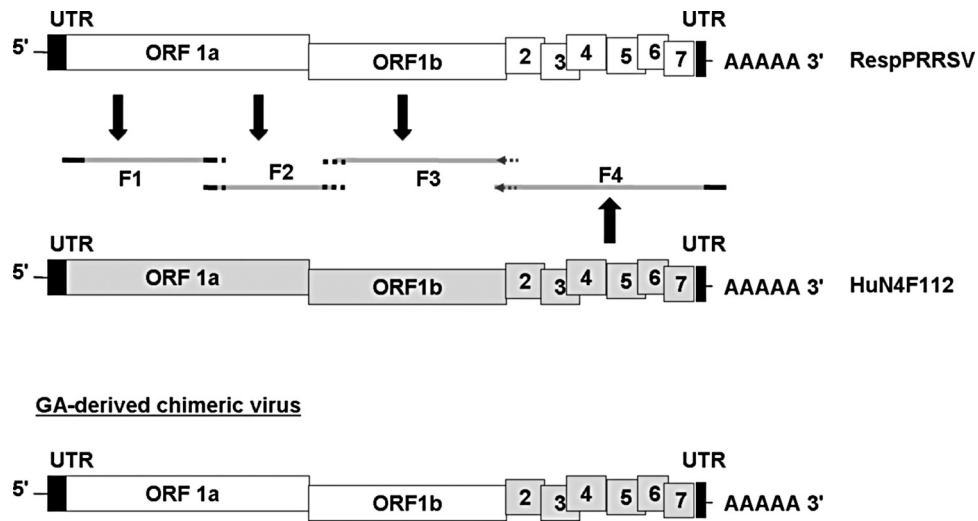


Fig. 5. The design used for generating chimeric virus. F1, F2, and F3 were RT-PCR amplified from the region covering 5' UTR until the end of ORF1ab of RespPRRSV. F4 was amplified from Hun4 F112 strain. The purified F1–F4 fragments were assembled into the modified pSMART BAC. After transfecting into a 293T-MARC-145 coculture, a chimeric virus displaying ORF1ab of RespPRRSV and ORFs 2–7 of Hun4 F112 was obtained.

3.5. Generation of a chimeric virus using the GA approach

Once it was established that GA could be used as a simple and quick method to generate a virus that resembled the parental virus, the possibility of using GA to generate a chimeric virus was examined. To create a chimeric virus, three cDNA fragments, F1, F2, and F3, derived from RespPRRSV vRNA and the F4 fragment amplified from the vRNA of the Hun4 F112 strain were used (Fig. 5). Using the same GA protocol described above, an infectious chimeric virus was generated and verified by restriction fragment

length polymorphism on two regions of the genome: the 5' region corresponding to the F1 fragment, and the 3' region corresponding to the F4 fragment. As shown in Fig. 6A, the digested 5' product of the chimeric virus showed a pattern similar to that of RespPRRSV, and was distinguishable from that of the Hun4 F112 strain. On the other hand, the digested 3' product of the chimeric virus displayed a pattern resembling the digested Hun4 F112 3' product, both of which differed from the RespPRRSV pattern, suggesting successful generation of a chimeric virus containing the 5' sequence of the RespPRRSV strain, and the 3' sequence of the Hun4 F112 strain.

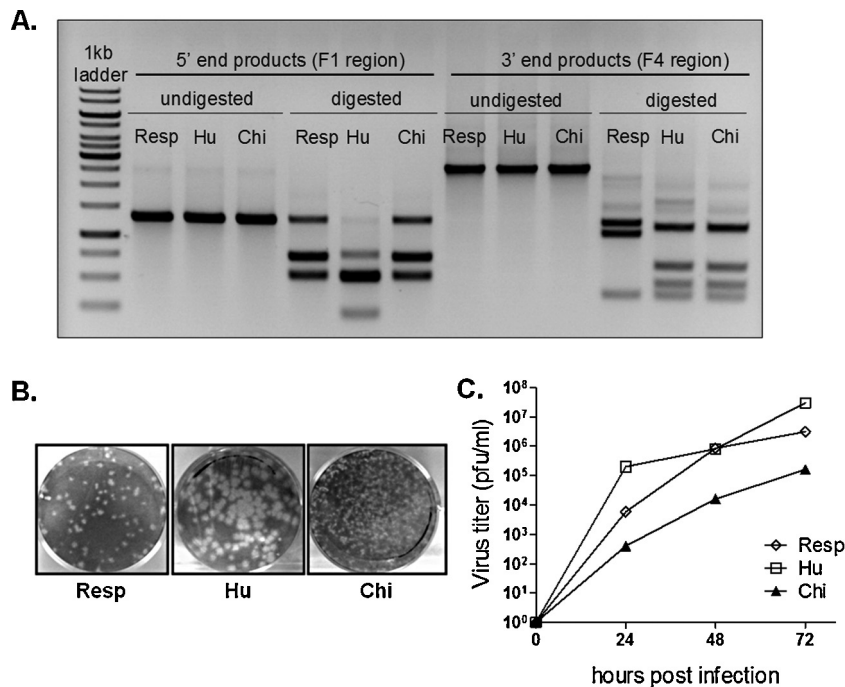


Fig. 6. The characterization of the GA-derived chimeric virus. (A) vRNAs were extracted from RespPRRSV (Resp), Hun4 F112 (Hu), or the GA-derived chimeric virus (Chi). Primers specific for a small region of F1 were used to obtain DNA products representing the 5' end of the genome, and primers specific for a small region of F4 were used to generate DNA products representing the 3' end of the genome. 5' end products were digested with AatI and NaeI, while 3' end products were digested with EcoRV and NruI. Undigested and digested products were analyzed on a 1% agarose gel. (B and C) MARC-145 cells were infected with RespPRRSV (Resp), Hun4 F112 (Hu), or the GA-derived chimeric virus (Chi). (B) The infected cells were overlaid with medium containing 0.9% agar. On day 4 post-infection, the cells were stained with crystal violet, and plaque sizes were documented. (C) Culture supernatants were collected every 24 h post-infection and the virus titers were measured by plaque assay.

Generation of chimeric viruses is useful in studying PRRSV biology and viral determinants of virulence. The chimeric virus generated here contains RespPRRSV F1–F3 fragments, which encode for non-structural ORF1ab gene, and Hun4 F112 F4 fragment, which encodes for structural ORFs 2–7 (Fig. 5). Therefore, the chimeric virus can be used to study the role of structural genes on viral growth characteristics. Examination of plaque sizes in MARC-145 cells showed that this chimeric virus displayed a small plaque size similar to the RespPRRSV strain, unlike the Hun4 F112 strain which exhibited a much larger plaque size (Fig. 6B). This suggests that ORF1ab, rather than ORFs 2–7, may play a more important role in determining the plaque size of the virus. Growth kinetics of the chimeric virus was also examined. As shown in Fig. 6C, the chimeric virus showed slower growth kinetics compared to the parental RespPRRSV or Hun4 F112, suggesting that non-structural genes originating from the RespPRRSV may not be compatible to the structural genes from the Hun4 F112. Altogether, these results highlight the efficacy of GA in studying PRRSV biology. Chimeric viruses containing genes of different viral strains can be conveniently generated and used to examine roles of viral genes in growth characteristics and virulence.

4. Discussion

For most viruses, infectious clone generation usually involves tedious, multi-step assembly of genomic fragments. Besides the length of time required and the possibility of introducing undesired mutations during the amplification of plasmid DNA in bacteria, the conventional bacterial cloning method may also encounter a problem with toxicity of certain viral sequences in the bacterial host. Many genomic cDNAs can be cloned successfully, but there are others such as the genomic cDNA of coronaviruses, nairoviruses and togaviruses that seem to be toxic when expressed in *Escherichia coli* (Bridgen, 2012; Ulper et al., 2008). With GA, potential cytotoxicity to the bacterial host is eliminated. Furthermore, the GA method utilizes overlapping sequences in the DNA fragments to link the DNA fragments to each other, eliminating the need to add or alter sequences for restriction enzyme sites to accommodate ligation. Introduction of any foreign sequences that may alter function of the viral genes can be minimized (Siridechadilok et al., 2013). GA thus provides an easy method to assemble the entire genome of a virus.

In this study, the RespPRRSV genomic sequence was assembled *in vitro* via the GA method. The 15 kb genome was amplified in four fragments with sizes between 3 and 5 kb. This range of cDNA fragment sizes was selected for ease in amplifying sequences and to minimize the number of fragments added to the GA reaction. As it was previously reported that increasing the number of DNA fragments in the GA reaction negatively affected assembly efficiency (Gibson et al., 2009, 2010), amplification of two 8 kb cDNA fragments from vRNA was attempted. However, the small amount of cDNA obtained was insufficient for GA reactions. Therefore, amplification of smaller pieces of cDNA was chosen and proven to be more suitable for achieving sufficient amounts of DNA for assembly. This study's design for reconstructing the PRRSV genome using GA had an F4 fragment spanning all the structural genes, making it convenient to generate chimeric viruses that contain foreign structural genes. However, the generation of chimeric viruses *via* this method is not restricted to this design. As the size of DNA fragments used in GA reactions can be varied, a variety of chimeric viruses derived from different lengths of cDNA fragments can be obtained. Thus one can conveniently utilize the GA method to customize the generation of chimeric viruses.

Following *in vitro* assembly of cDNA fragments, DNA products larger than the input DNA fragments were formed, indicating that

the overlapping primers and the assembly protocols were designed properly. However, a significant amount of the original fragments still remained unassembled after the GA reactions were completed (Fig. 2). This may be due to excess amounts of particular fragments or suboptimal amounts of enzymes in the home-made master mix. It is also possible that the GA reaction is intrinsically inefficient since the presence of non-assembled DNA fragments was also reported in the original study describing the GA protocol (Gibson et al., 2008, 2009).

GA is an efficient platform to recover PRRSV *in vitro*. By transfecting the GA reaction mixture containing the viral cDNA fragments, infectious particles were readily recovered. The transfected culture supernatant collected on day 3 post-transfection was infectious and caused CPE in the subsequent infection of MARC-145 cells. The observed incubation time for the virus recovery appeared similar to that of another reported system similarly utilizing a CMV promoter to generate vRNA in transfected cells (Wang et al., 2013). It is, however, slightly slower than a virus recovery system utilizing a direct transfection of T7 promoter-driven, *in vitro* transcribed vRNA into MARC-145 cells. The culture supernatant of the later system was reported to be infectious by 24 h post-transfection (Zhang et al., 2011). Thus, the CMV-driven system may require a longer incubation time than a direct transfection of vRNA. In agreement with these previous reports, we also observed more extensive CPE in vRNA transfected than the GA mixture transfected 293T-MARC-145 cocultures (data not shown) and in the subsequent infection of MARC-145 cells (Fig. 3A). While the CMV-driven system may require longer time to recover infectious PRRSV, the elimination of the *in vitro* transcription step provides a clear advantage of using GA in generating PRRSV particles.

As demonstrated here, GA can be used to generate chimeric viruses which are useful not only for studying PRRSV biology but also for the development of novel PRRSV vaccines. One can amplify the corresponding F4 fragment from a field isolate and combine it with cDNA fragments derived from a vaccine strain. The resulting virus may be used as a vaccine candidate containing structural genes that match virus found in the field. Such a strategy was examined in our laboratory and an infectious chimeric virus was generated in the 293T-MARC-145 co-culture transfected with a GA reaction mixture. Unfortunately, the chimeric virus was not readily grown to a high titer in subsequent passages in MARC-145 cells. The virus needed several passages in MARC-145 cells before reaching a high titer (data not shown). This finding highlights the need to further improve the rescue protocol for viruses obtained from the field. It remains to be tested whether modified cell lines, such as PK15 or BHK-21 constitutively expressing CD163 shown to enhance the growth of PRRSV (Calvert et al., 2007; Delrue et al., 2010), can be used to improve the virus recovery *via* the GA method.

5. Conclusion

Infectious GA-derived RespPRRSV was successfully generated *in vitro*. The rescued virus displayed characteristics that resembled the parental virus, suggesting that GA can be further customized to generate chimeric viruses of interest. The GA protocol was then used to create a chimeric virus that contained the non-structural ORF1ab gene from one strain and the structural ORFs 2–7 genes from another. Because the GA reaction utilizes overlapping DNA sequences in the assembly process, it eliminates the need to alter viral sequences to accommodate restriction enzyme sites needed for conventional cloning of viral molecular clones. The protocol described here is thus valuable as it serves as a quick and efficient platform to make desired viruses and is immediately applicable to the study of PRRSV biology and vaccine development.

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References

- Almazan, F., DeDiego, M.L., Sola, I., Zuniga, S., Nieto-Torres, J.L., Marquez-Jurado, S., Andres, G., Enjuanes, L., 2013. Engineering a replication-competent, propagation-defective Middle East respiratory syndrome coronavirus as a vaccine candidate. *MBio* 4 (5), e00650–e713.
- Almazan, F., Gonzalez, J.M., Penzes, Z., Izeta, A., Calvo, E., Plana-Duran, J., Enjuanes, L., 2000. Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. *Proc. Natl. Acad. Sci. U. S. A.* 97 (10), 5516–5521.
- Bridgen, A., 2012. *Reverse Genetics of RNA Viruses: Applications and Perspectives*. John Wiley & Sons, Chichester, West Sussex.
- Calvert, J.G., Slade, D.E., Shields, S.L., Jolie, R., Mannan, R.M., Ankenbauer, R.G., Welch, S.K., 2007. CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. *J. Virol.* 81 (14), 7371–7379.
- Delrue, I., Van Gorp, H., Van Doorselaere, J., Delputte, P.L., Nauwynck, H.J., 2010. Susceptible cell lines for the production of porcine reproductive and respiratory syndrome virus by stable transfection of sialoadhesin and CD163. *BMC Biotechnol.* 10, 48.
- Fang, Y., Rowland, R.R., Roof, M., Lunney, J.K., Christopher-Hennings, J., Nelson, E.A., 2006. A full-length cDNA infectious clone of North American type 1 porcine reproductive and respiratory syndrome virus: expression of green fluorescent protein in the Nsp2 region. *J. Virol.* 80 (23), 11447–11455.
- Gibson, D.G., Benders, G.A., Andrews-Pfannkoch, C., Denisova, E.A., Baden-Tillson, H., Zaveri, J., Stockwell, T.B., Brownley, A., Thomas, D.W., Algire, M.A., Merryman, C., Young, L., Noskov, V.N., Glass, J.L., Venter, J.C., Hutchison 3rd, C.A., Smith, H.O., 2008. Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. *Science* 319 (5867), 1215–1220.
- Gibson, D.G., Smith, H.O., Hutchison 3rd, C.A., Venter, J.C., Merryman, C., 2010. Chemical synthesis of the mouse mitochondrial genome. *Nat. Methods* 7 (11), 901–903.
- Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison 3rd, C.A., Smith, H.O., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* 6 (5), 343–345.
- Huang, Y.W., Meng, X.J., 2010. Novel strategies and approaches to develop the next generation of vaccines against porcine reproductive and respiratory syndrome virus (PRRSV). *Virus Res.* 154 (1–2), 141–149.
- Kimman, T.G., Cornelissen, L.A., Moormann, R.J., Rebel, J.M., Stockhofe-Zurwieden, N., 2009. Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. *Vaccine* 27 (28), 3704–3718.
- Lee, C., Calvert, J.G., Welch, S.K., Yoo, D., 2005. A DNA-launched reverse genetics system for porcine reproductive and respiratory syndrome virus reveals that homodimerization of the nucleocapsid protein is essential for virus infectivity. *Virology* 331 (1), 47–62.
- Meulenbergh, J.J., Bos-de Ruijter, J.N., Wensvoort, G., Moormann, R.J., 1998. An infectious cDNA clone of porcine reproductive and respiratory syndrome virus. *Adv. Exp. Med. Biol.* 440, 199–206.
- Nielsen, H.S., Liu, G., Nielsen, J., Oleksiewicz, M.B., Botner, A., Storgaard, T., Faaberg, K.S., 2003. Generation of an infectious clone of VR-2332, a highly virulent North American-type isolate of porcine reproductive and respiratory syndrome virus. *J. Virol.* 77 (6), 3702–3711.
- Opriessnig, T., Halbur, P.G., Yoon, K.J., Pogranichniy, R.M., Harmon, K.M., Evans, R., Key, K.F., Pallares, F.J., Thomas, P., Meng, X.J., 2002. Comparison of molecular and biological characteristics of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (ingelvac PRRS MLV), the parent strain of the vaccine (ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. *J. Virol.* 76 (23), 11837–11844.
- Rossow, K.D., 1998. Porcine reproductive and respiratory syndrome. *Vet. Pathol.* 35 (1), 1–20.
- Siridechadilok, B., Gomutsukhavadee, M., Sawaengpol, T., Sangiambut, S., Puttikhunt, C., Chin-inmanu, K., Suriyaphol, P., Malasit, P., Sreaton, G., Mongkolsapaya, J., 2013. A simplified positive-sense-RNA virus construction approach that enhances analysis throughput. *J. Virol.* 87 (23), 12667–12674.
- Tian, Z.J., An, T.Q., Zhou, Y.J., Peng, J.M., Hu, S.P., Wei, T.C., Jiang, Y.F., Xiao, Y., Tong, G.Z., 2009. An attenuated live vaccine based on highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) protects piglets against HP-PRRS. *Vet. Microbiol.* 138 (1–2), 34–40.
- Ulper, L., Sarand, I., Rausalu, K., Merits, A., 2008. Construction, properties, and potential application of infectious plasmids containing Semliki Forest virus full-length cDNA with an inserted intron. *J. Virol. Methods* 148 (1–2), 265–270.
- Wang, C., Huang, B., Kong, N., Li, Q., Ma, Y., Li, Z., Gao, J., Zhang, C., Wang, X., Liang, C., Dang, L., Xiao, S., Mu, Y., Zhao, Q., Sun, Y., Almazan, F., Enjuanes, L., Zhou, E.M., 2013. A novel porcine reproductive and respiratory syndrome virus vector system that stably expresses enhanced green fluorescent protein as a separate transcription unit. *Vet. Res.* 44, 104.
- Yun, S.I., Lee, Y.M., 2013. Overview: replication of porcine reproductive and respiratory syndrome virus. *J. Microbiol.* 51 (6), 711–723.
- Zhang, S., Zhou, Y., Jiang, Y., Li, G., Yan, L., Yu, H., Tong, G., 2011. Generation of an infectious clone of HuN4-F112, an attenuated live vaccine strain of porcine reproductive and respiratory syndrome virus. *Viol. J.* 8, 410.