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Rapid reconstruction of porcine reproductive and respiratory syndrome virus using synthetic DNA fragments

Julien Mélade [a,*], Géraldine Piorkowski [a], Hawa Sophia Bouzidi [a,b], Alain Medawar [b], Claudine Raffy [b], Xavier de Lamballerie [a], Antoine Nougairède [a]

[a] Unité des Virus Émergents (UVE: Aix-Marseille Univ-IRD 190-Inserm 1207), Marseille, France
[b] VIRBAC, 1e Avenue, 13ème rue, LID, BP27 - 06511 Carros, France

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most challenging infectious disease of pig populations causing devastating economic loss to swine industry. Reverse genetics allow to engineer modified viruses such attenuated strains for vaccine development. Some reverse genetic systems were described for PRRSVs but, due to genome complexity of PRRSVs, construction and modification of such systems remain laborious and time-consuming.

In this study, we described a reverse genetics approach based on the "Infectious-Subgenomic Amplicons" (ISA) method to rescue infectious PRRSV particles. Permissive cells were transfected with 4 overlapping synthetic DNA fragments covering the entire genome of PRRSV which allowed the rapid reconstruction of the complete virus genome and the subsequent generation of infectious wild-type particles within days.

The ISA method represent a rapid alternative of conventional reverse genetic systems. This method will help to generate genetically modified and attenuated strains for the development of sanitary countermeasures in the future.

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, positive-stranded RNA virus (Order: *Nidovirales*; Family: *Arteriviridae*) which belongs to *Betaarterivirus suid 1* and *Betaarterivirus suid 2*, formely European and North-American genotypes respectively [14,31,41,53]. PRRSV genome length is around 15 kb and consists of at least 10 open reading frames (ORFs): ORF1a/ORF1ab, occupy approximately the three-quarters of the genome and encodes for several non-structural proteins (nsp) followed by ORF2a/ORF2b, ORF3, ORF4, ORF5/ORF5a, ORF6 and ORF7 [27] . ORF1a and ORF1ab polyproteins are involved in RNA replication, ORF2a/2b encodes for glycoprotein 2 (GP2) and envelope protein (E), ORF3 for GP3 ORF4 for GP4 which all together are involved in virus's entry [9]. ORF5/ORF5a encodes for GP5 and GP5a [37], ORF6 codes for the membrane (M), and ORF7 for the nucleocapsid protein (N) [20].

PRRSV is the causative agent of porcine reproductive and respiratory syndrome in swine populations. Infection leads to reproductive failure in sows and severe respiratory disease in newborn and growing pigs. PRRSV continuously struggle the global swine industry by causing devastating economic losses, of approximately $664 million per year in the United States [32] and the emergence of genetically divergent strains in Asia has led to highly pathogenic PRRSVs (HP-PRRSVs) [45,2]. Vaccines against PRRSV exist and confer protection from the disease [36], however, vaccination remains a challenge, considering the high antigenic heterogeneity of current circulating viral strains [23]. Indeed, the efficacy of licensed vaccines to protect against both genotypes of PRRSV is still unclear [17,38,39,41] .

Reverse genetics allow the engineering of wild-type and genetically modified viruses and has greatly help to elucidate the viral biological properties and engineer attenuated strains of RNA viruses for vaccination strategies [43]. In the case of PRRSV, rescue of infectious viruses may be obtained through DNA-based or RNA-based strategies : in the first approach, viruses can be rescue by the direct transfection of a full-length cDNA clone under the control of the human cytomegalovirus promoter (pCMV) [24,18,33,44]; in the

* Corresponding author at: Faculté de Médecine de Marseille 27, Bd Jean Moulin, 13005 Marseille, France.
E-mail address: julien.melade@univ-amu.fr (J. Mélade).

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second approach, a full-length cDNA is cloned downstream of a bacteriophage RNA polymerase (T7 or SP6), to produce after *in vitro* transcription, a full-length viral RNA transcripts [12,18,24,28,33,46]. Due to genome length and complexity of PRRSV, construction of full-length cDNA clone and modification of recombinant strains remain laborious and time-consuming. In this context, swift and simple reverse genetic tools represent an advantage.

In recent years, a rapid reverse genetics method was developed to rescue (+) ssRNA viruses from *Flaviviridae*, *Togaviridae* and *Picornaviridae* families and recently *Coronaviridae* family [4,5,26]: the Infectious Subgenomic Amplicons (ISA) method. Unlike conventional reverse genetic systems that require cloning steps, this method is based on the simple transfection of permissive cells with overlapping subgenomic cDNA fragments, derived from synthetic DNA fragments and covering the entire virus genome. This method allowed the rapid and fluent generation of genetically modified viruses [5].

In the current study, we applied the ISA method to rescue, within days, wild-type and infectious PRRSV particles. Genotypic and phenotypic characterization of recovered viruses were performed using the original isolate for comparison. The ISA method will be used to rapidly develop genetically modified PRRSV strains in the future.

2. Material and methods

Cells. MARC-145 cells expressing the sialoadhesine receptor CD-169 (MARC-Sn cells) were grown in MEM, GlutaMAX (Thermo Fisher Scientific) with 10% heat-inactivated foetal calf serum (FCS; Life Technologies), 1% penicillin/streptomycin (P/S; 10,000 U/mL; 10 mg/mL; Thermo Fisher Scientific) and 0.5% gentamycin (10000 U/mL; Thermo Fisher Scientific). Baby Hamster Kidney 21 cells (BHK-21; ATCC CCL-10) were grown in Minimum Essential Media (MEM; Thermo Fisher Scientific) with 5% FCS, 1% L-glutamine (200 Mm; Thermo Fisher Scientific), 5% Tryptose Phosphate Broth (TPB; Thermo Fisher Scientific) and 1% P/S. MARC-Sn cells were kindly provided by Dr. Hans Nauwynck (University Ghent, Belgium).

Viral strains. A PRRSV original isolate was provided by the VIRBAC laboratory (Genbank accession number: MW366748). PRRSV original isolate was firstly passaged 5 times on MARC-Sn cells. An MOI of 0.1 was used to infect a 12.5 cm2 culture flasks of confluent MARC-Sn cells with the original isolate. Cells were washed twice (HBSS) 1 h after the infection and 4 mL of medium was added. Cell supernatant media was sampled at 72 h post-infection, clarified by centrifugation, aliquoted and stored at − 80 °C. All manipulations were performed in a biosafety level 3 (BSL3) containment.

Virus full genome sequencing. Clarified cell supernatant was used to determine full viral genome sequences. Extraction of nucleic acid was performed using the EZ1 advanced XL machine (Qiagen) with the EZ1 Virus Mini Kit v2.0 (Qiagen). The first random amplification of the viral genome nucleic acids was performed as previously described [5]. Amplification was also performed using specific primers and the RT-PCR kit. The SuperScript™ III One-Step RT-PCR System with Platinum Taq High fidelity DNA polymerase kit (Thermo Fisher Scientific) was used. Sequences of primers designed from the full-length sequences are available in table S1. PCR products were purified (Monarch® PCR & DNA Cleanup Kit; New England Biolabs) and pooled in equimolar proportions. After Qubit quantification using Qubit® dsDNA HS Assay Kit and Qubit 2.0 fluorometer (Thermo Fisher Scientific), amplicons were fragmented (sonication) into fragments of 200 bp long. Libraries were built by adding barcodes, for sample identification and primers to fragmented DNA using AB Library Builder System (Thermo Fisher Scientific). To pool equimolarly the barcoded samples a quantification step by real time PCR using Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific) was performed. Emulsion PCR of the pools and loading on a 530 chip was realised using the automated Ion Chef instrument (Thermo Fisher Scientific). Sequencing was performed using the S5 Ion torrent technology v5.12 (Thermo Fisher Scientific) following the manufacturer's instructions. Consensus sequence was obtained after trimming of reads (reads with quality score < 0.99, length < 100pb were removed and the 30 first and 30 last nucleotides were removed from the reads) mapping of the reads on a reference (determined following blast of De Novo contigs) using CLC genomics workbench software v.20 (Qiagen). *De novo* contigs were produced to ensure that the consensus sequence was not affected by the reference sequence. Mutations with frequencies > 50% were defined as majority mutations in sequencing reads. Mutations with frequencies between 5% − 50% in sequencing reads were defined as minority mutations.

Alignment and phylogenetic analysis. Amino acids deletion in Nsp2 were identified using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) alignment with default parameters in Geneious Prime® 2021.1.1 [22]. To determine the PRRSV lineage of the original isolate, a Multiple Alignment using Fast Fourier Transform (MAFFT) alignment was performed using 47 reference sequences collected in Genbank and belonging to lineage 1 from 9 [49,50]. Phylogenetic trees were constructed using MrBayes 3.2.6 [19] in Geneious Prime, rooted with a Lelystad virus sequence (GenBank number NC043487). A minimum of two independent runs were made, with four chains in each run, for a total of 5 000 000, sampling every 5000 generations. The first 5000 trees burn-in were discarded. The obtained effective sample size values (ESS) for each parameter were all superior to 200. Trees obtained after the convergence point were summarized and visualized by FigTree 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Preparation of subgenomic cDNA fragments. Four overlapping synthetic DNA fragments were designed *in silico* and supplied by the manufacturer (Genscript manufacturers). The first and last fragments were directly flanked at their 5′ and 3′extremities by the human cytomegalovirus promoter (pCMV) and the hepatitis delta virus ribozyme followed by the simian virus 40 polyadenylation signal (HDR/SV40pA) (see supplemental text a and b) respectively during *de novo* synthesis. cDNAs were generated by PCR using these *de novo* synthetic DNA plasmids as templates. PCR amplifications were performed using the Super Fidelity PCR polymerase kit (Thermo Fisher Scientific). Primer sequences are described on table S2. The final mixture contained 25 μL of reaction mix, 2 μL of DNA (1 ng/μL), 100 nM of each primer and 20 μL nuclease-free water. PCR reactions were performed on a Biometra TProfessional Standard Gradient thermocycler: with the following conditions: 98 °C for 30 s followed by 35 cycles of 98 °C for 10 s, for 58 °C for 10 s, 72 °C for 30 s/kb and 72 °C for 5 min. Amplicons were purified (Monarch® PCR & DNA Cleanup Kit; New England Biolabs) and the size of PCR products was verified by gel electrophoresis. The integrity of each DNA fragment was confirmed by sequencing before transfection.

Cell Transfection. The four cDNA fragments were equimolarly pooled (~75 ng/fragment; final concentration of 300 ng) with Lipofectamine 3000 (Thermo Fischer Scientific) for 45 min and transfected into a coculture of BHK-21 + MARC-Sn cells. Fresh medium was added 24 h after transfection and plates were re-incubated for 5 days at 37 °C with 5% CO2. After 5 days, cell supernatant was harvested, centrifuged and serially passaged 2 times in MARC-Sn cells to ensure the complete disappearance of the DNA used during the transfection. Each passages were performed by inoculating undiluted clarified supernatant media

onto subconfluent Marc-Sn cells: after 1 h of incubation, cells were washed twice using Hanks' Balanced Salt solution (HBSS; Gibco) fresh medium was added and cellswere incubated 3 days. After the 2nd passage, cell supernatant media were harvested, clarified by centrifugation, aliquoted and stored at −80 °C. These virus stocks were used to perform quantification of viral RNA, $TCID_{50}$ assay, kinetic growth and whole-genome sequencing.

Real time RT-PCR assay for virus detection. Viral RNA was extracted from 100 µL of cell supernatant using a QIAamp Viral RNA kit and RNase-Free DNase Set on the automated QIAcube (Qiagen), following manufacturer's instructions. Relative quantification of viral RNA was performed using the express One-Step SuperScript® qRT-PCR (Invitrogen). The mixture contained 5 µL of express qPCR SuperMix Universal, 0.25 µL of each primer (500 nM), 0.1 µL of probe (200 nM), 1 µL of express SuperScript® RT mix and 3,5 µL of extracted nucleic acids. Assays were performed using the QuantStudio 12 K Flex Real-Time PCR machine (Life technologies) with the following conditions: 50 °C for 15 min, 95 °C for 20 sec, followed by 45 cycles of 95 °C for 3 s, 60 °C for 30 s. Data collection occurred during the 60 °C step. Synthetic RNA was used to calculated the amount of viral RNA from standard curves. Primers and probes used are described in the table S3.

Tissue Culture Infectious Dose 50 ($TCID_{50}$) assay. A 96-well plate culture of sub-confluent MARC-Sn cells was used. Cells were inoculated with 10-fold serial dilutions of clarified cell supernatant media and incubated 4 days. Each row included 6 wells of the dilution and two negative control. A following direct immunofluorescence assay (dIFA) was performed. The determination of the $TCID_{50}$/mL for both viruses was performed using the Reed and Muench method [35].

Direct immunofluorescence assay (dIFA). The nucleocapsid (N) protein expression in the ISA strain and original isolate was determined as followed : cells were washed with PBS, fixed 20 min with 100% cold methanol, washed again with PBS, incubated with an appropriately diluted mouse anti-nucleocapside anti-PRRSV monoclonal antibody 13E2 (mAb 13E2; kindly provided by Dr. Hans Nauwynck, Ghent University, Belgium) for 1 h at 37 °C. Cells were rinsed two times with PBS, and incubated with a goat anti-mouse IgG FITC antibody (Jackson Immunoresearch) diluted at 1:100 for 1 h at 37 °C. Plate were washed with PBS and read for presence or absence of the N protein using a DMI8 Leica fluorescence microscope.

Virus replication kinetics. An MOI of 0.01 was used to infect a 12.5 cm2 culture flask of confluent MARC-Sn cells with the ISA or PRRSV original isolate . Cells were washed twice (HBSS) 1 h after the infection and 4 mL of medium was added. Four hundred µL of cell supernatant media were sampled every 12 h up to 72 h post-infection, clarified by centrifugation, aliquoted and stored at − 80 °C. They were then analyzed using the real-time RT-PCR assay or $TCID_{50}$ assay as described above.

Nucleotide sequence accession numbers. The complete genome sequence of the original isolate sequenced at the first passage have been deposited in GenBank under the reference number MW366748.

Statistical analyses. Exploratory analysis was performed using a two-way ANOVA for multiple comparisons with Sidak's multiple comparisons test. Statistical analysis and graphical representation were performed using GraphPad Prism 7.00.

3. Results

Nucleotide alignment based on nsp2 showed a discontinuous 35 amino acid deletions in the original isolate, similarly to novel emerging PRRSV strains such as NADC30 and the HP-PRRSV JXA1 (Fig. 1). Phylogenetic analyses based on ORF5 indicated that the original isolate was grouped into lineage 8 in which branched well known HP-PRRSV strains (Fig. 2) [13].

To ensure an efficient replication in MARC-Sn cells, the PRRSV original isolate was firstly passaged five times. Complete genome sequencing of infectious cell supernatant media was performed at first and fifth passages. Comparison of both sequences revealed a total of six non-synonymous and one synonymous mutations located in the ORF1a/1ab, GP3 and GP4 coding regions of the complete virus genome (Table 1).

Based on the complete genome sequence of the original isolate at the fifth passage, the entire viral genome was *de novo* synthetized into 4 overlapping DNA fragments (Fig. 3). During *de novo* synthesis, the pCMV was added at the 5′ extremity of the first fragment and the HDR/SV40pA was added at the 3′ extremity of the last fragment. Generation of subgenomic cDNA fragments was performed by PCR using these *de novo* synthetized DNA fragments as templates. Co-culture of BHK-21 and MARC-Sn cells were transfected with cDNA fragments. After 5 days of transfection, supernatant were passaged 2 times on infected competent cells with

Fig. 1. Amino acid deletion in nsp2. A multiple alignment of PRRSV nsp2 was performed using MAFFT alignment. Deletion were labeled in blue boxes. Amino acid positions were indicated above the VR-2332 sequence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

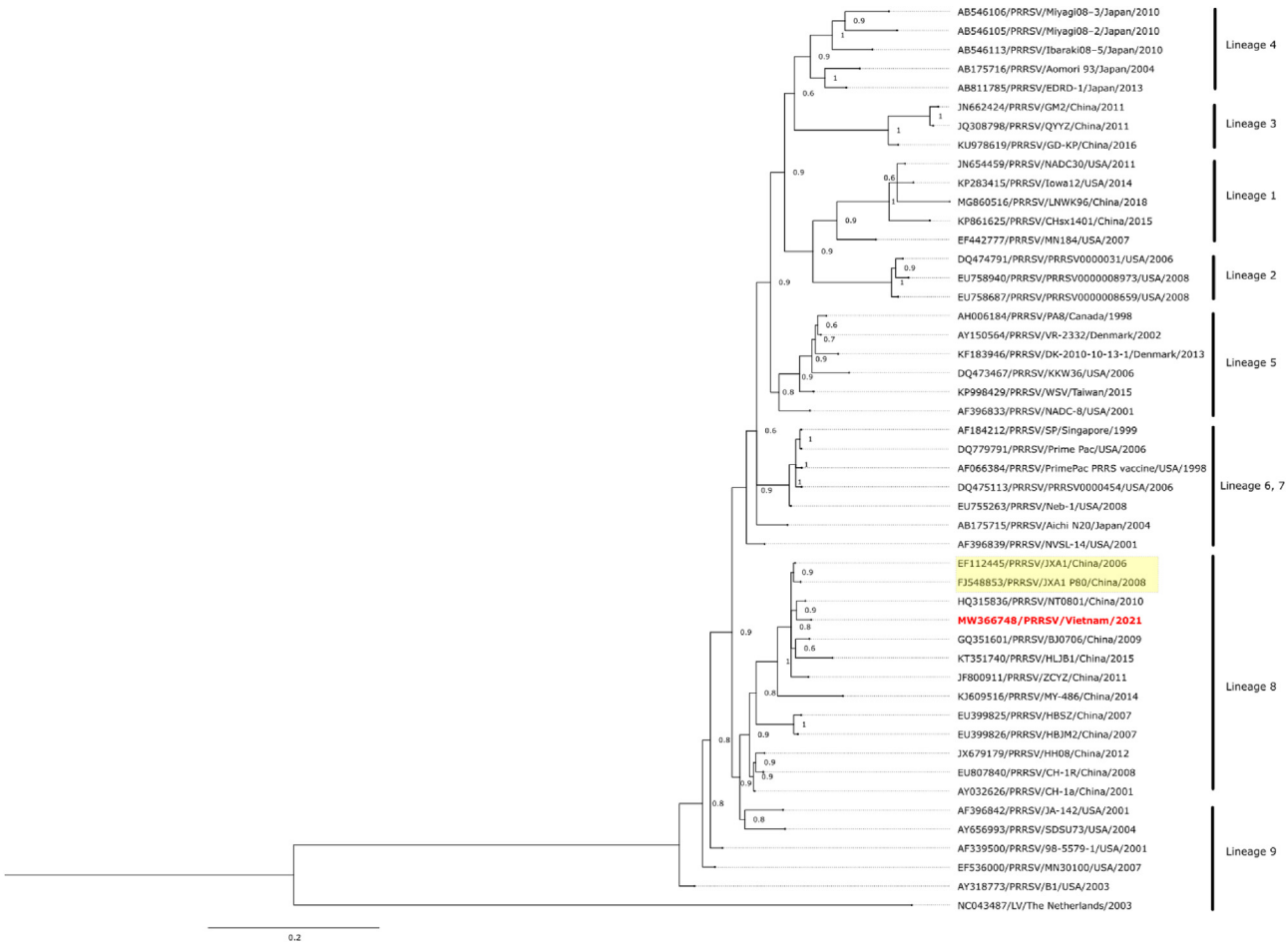


Fig. 2. Phylogenetic tree based on PRRSV ORF5. A global phylogeny of 47 PRRSV ORF5 sequences collected on Genbank and the sequence use in this study was calculated in 5,000,000 iterations in MrBayes with the GTR + G + I evolutionary model and a 10% burn-in rooted with a Lelystad virus sequence (GenBank number: NC43487). The sequence of the original isolate in this study was marked in bold red. Each lineage was indicated in the right. HP-PRRSV strains were highlight in yellow. Genbank accession number, virus name, strain name, country and date of isolation if available, were indicated in branch label. LV: Lelystad virus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Full-length sequencing of PRRSV original isolate. The original PRRSV isolate strain was passaged five times in Marc-Sn cells. Complete genome analyses were performed at the first and last passage. Characteristics of the mutations that emerged during passages are described for each virus. Nt: Nucleotide; AA: amino acid; GP: Glycoprotein; nsp: non-structural protein; "-": silent mutation.

Nt position	Region	AA position	Nt change	AA change
3012	ORF1a (nsp2)	941	G → T	M → I
3055	ORF1a (nsp2)	956	A → G	S → G
10,717	ORF1ab (nsp10)	1044	T → G	I → S
12,710	GP3	247	T → C	L → S
13,265	GP3	43	G → A	D → N
13,561	GP4	141	C → T	–
13,783	GP4	33	A → G	N → S

3 days of incubation at each passages. A successful rescue was demonstrated after two passages on MARC-Sn cells, using a combination of the following criteria: (i) detection of cytopathic effect (CPE), nucleocapsid protein expression by direct immunofluorescence, (ii) production of viral genomes in cell supernatant medium using real time qRT-PCR method, (iii) production of infectious particles in cell supernatant medium using $TCID_{50}$ assays, and (iv) complete viral genome sequencing using the NGS method. Except for NGS sequencing, each experiment was tested three times. For each transfection, two negative controls were performed: in the first one, only 3 cDNA fragments out of 4 were transfected; in the second one, no fragment was transfected. Before interpretation, we ensured that no CPE or viral genomes were detected in each of those controls.

We successfully rescue infectious particles of PRRSV as recorded by clear CPEs observed after 2 passages on MARC-Sn cells similarly to the original isolate (Fig. 4A). Direct immunnofluorescence assay showed a clear expression of the N protein in the ISA and original isolate in MARC-Sn cells (Fig. 4B). The production of infectious particles of the ISA strain was confirmed by the amounts of viral RNA load and infectious viral unit detected in supernatant media after two passages (7.0 +/- 0.1 log_{10} copies per mL and 5.3 +/- 0.4 log_{10} $TCID_{50}$ per mL respectively) (Table 2). In comparison to the original isolate, the amounts of viral RNA load recorded was 6.5 +/- 0.1

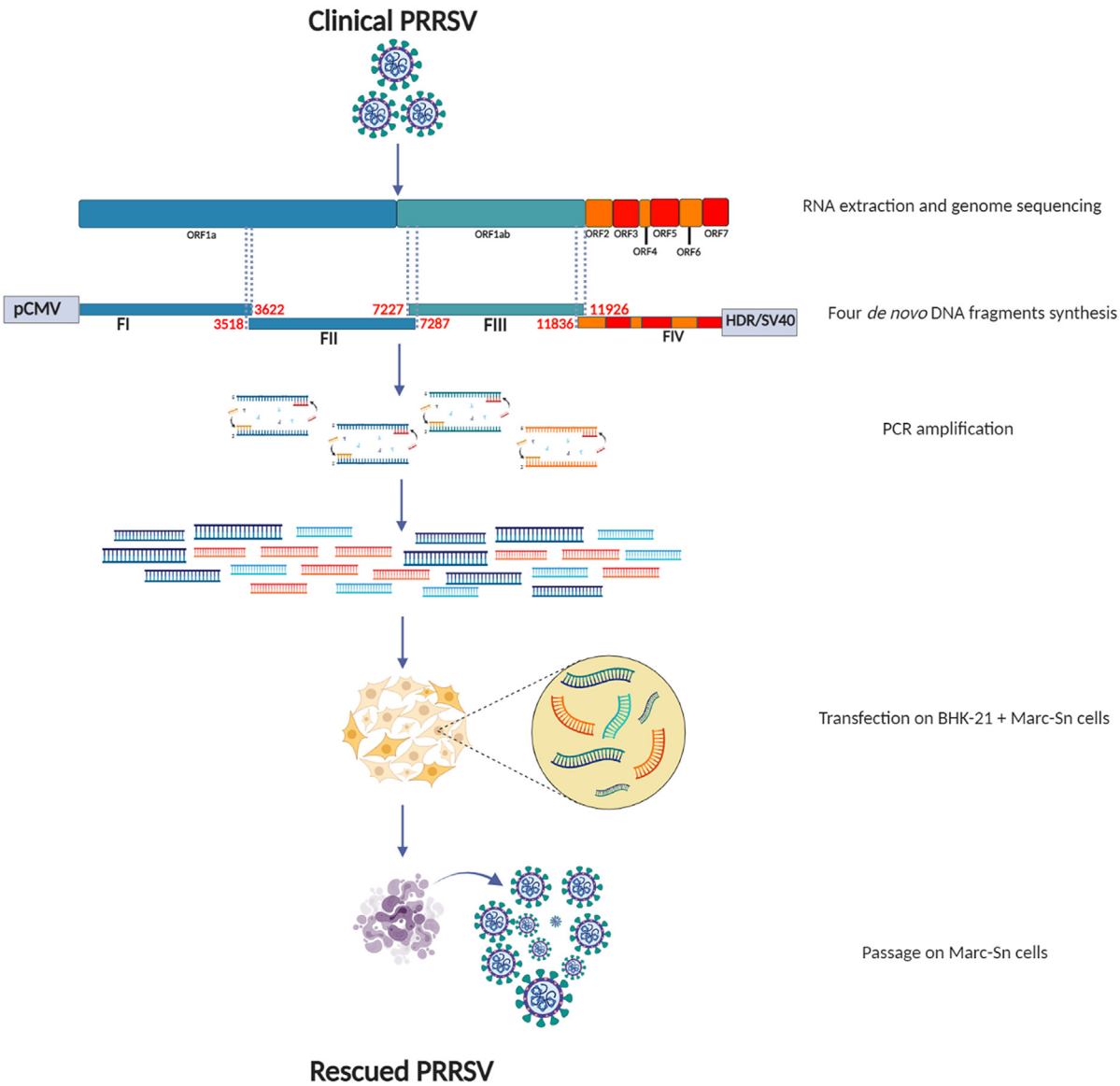


Fig. 3. Reverse genetics strategy used in this study(created with BioRender.com). The full-length sequence of PRRSV was used to design four overlapping synthetic DNA fragments covering the full genome of PRRSV. Positions on the genome (in nucleotide) are indicated in bold red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

log_{10} copies per mL and the infectious viral unit was 6.6 +/- 0.6 log_{10} $TCID_{50}$ per mL (Table 2) .

Comparative replication kinetics with rescued virus and original isolate was performed to ensure replicative fitness similarity. Cells were infected at MOI of 0.01, supernatant media were harvested every 12 h and amounts of viral RNA were measured using a real-time RT-qPCR and TCID50 assays to determine the amount of viral RNA or infectious viral unit respectively (Fig. 5A and 5B). Replication kinetic displayed very similar shapes despite one significant difference in viral RNA copies observed at 36 h post-infection between rescued virus and original isolate (4.9 +/- 0.1 vs 5.9 +/- 0.6 log10 RNA copies/mL; Fig. 5A) concluding that both ISA strain and original isolate exhibited similar replicative fitness in cell culture.

Complete genomic sequencing of the ISA strain revealed two majority mutations and three minority mutations in ORF1a and ORF1ab coding region respectively (Table 3). The genome integrity of the rescued virus was preserved at 99.98%.

4. Discussion

The continual circulation and emergence of novel PRRSV strains represent a real challenge to the swine industry worldwide. In this study, we started with an *Betaarterivirus suid 2* original isolate. We successfully developed a rapid and bacterium-free reverse genetics system to swiftly reconstruct wild-type and infectious particles of PRRSV. Viral particles closely recapitulate the phenotype, in cell culture, and the genotype of the original isolate.

Complete genome sequencing followed by phylogenetic classification of the original strain demonstrated a particular genetic pattern in nsp2 observed in novel emerging PRRSV strains [15] and a close phylogenetic clusterisation with HP-PRRSV strains. Interestingly, after serial passages in MARC-Sn cells, we recorded 6 non-synonymous mutations and one silent mutation distributed over nsp2, nsp10, GP3 and GP4 of the original isolate. Viral adaptation in cell culture is often associated with changes in the consensus sequence and emergence of mutant spectrum which may

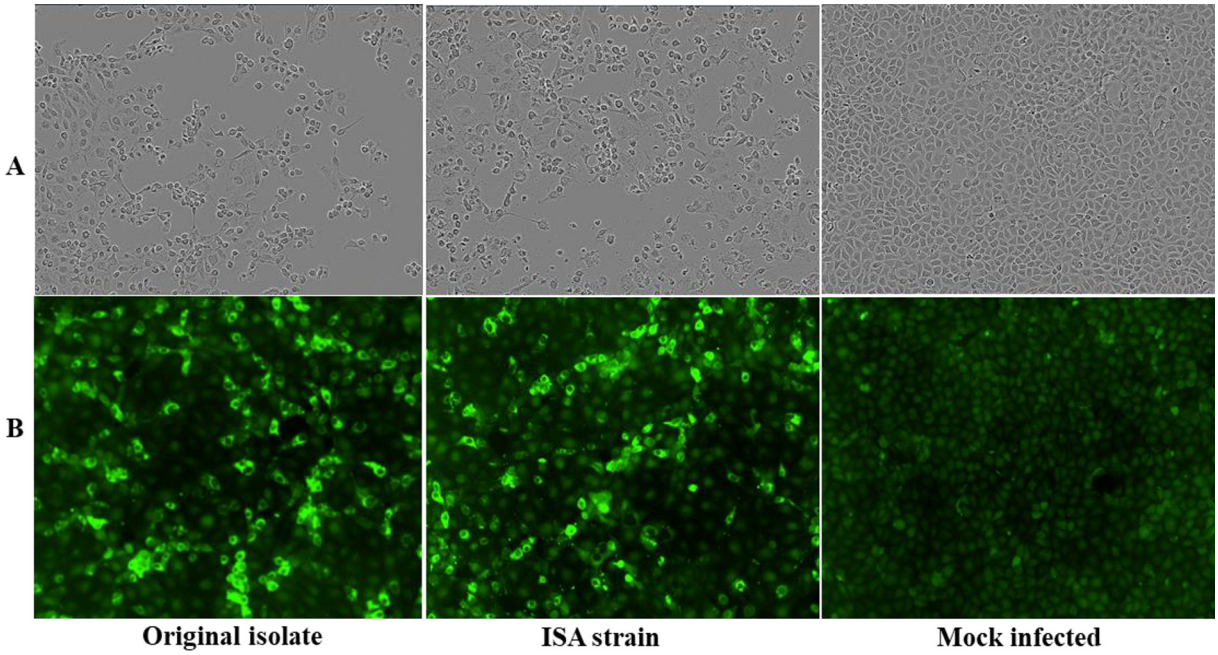


Fig. 4. Microscopy analysis of PRRSV original isolate and ISA strain on Marc-Sn infected cells. Marc-Sn cells were infected with an MOI of 0.01 with the original isolate, the ISA strain or mock infected. Cytopathic effect (A) and dIFA for N expression (B) were recorded at 72 h pi.

Table 2
Phenotypic characterization of original isolate and ISA PRRSV. For PRRSV original isolate and ISA strain, cell lines used for transfection and passage, presence of cytopathic effect (CPE), direct immunoassay for PRRSV nucleocapsid, quantification of viral RNA and infectious titers in cell supernatant media are summarized. Each experiment was performed in triplicate. The infectious titers and amount of RNA copies were expressed as log_{10} $TCID_{50}$/ml and log_{10} copies/ml respectively (mean +/- SD). Data are representative of at least three independent experiments.

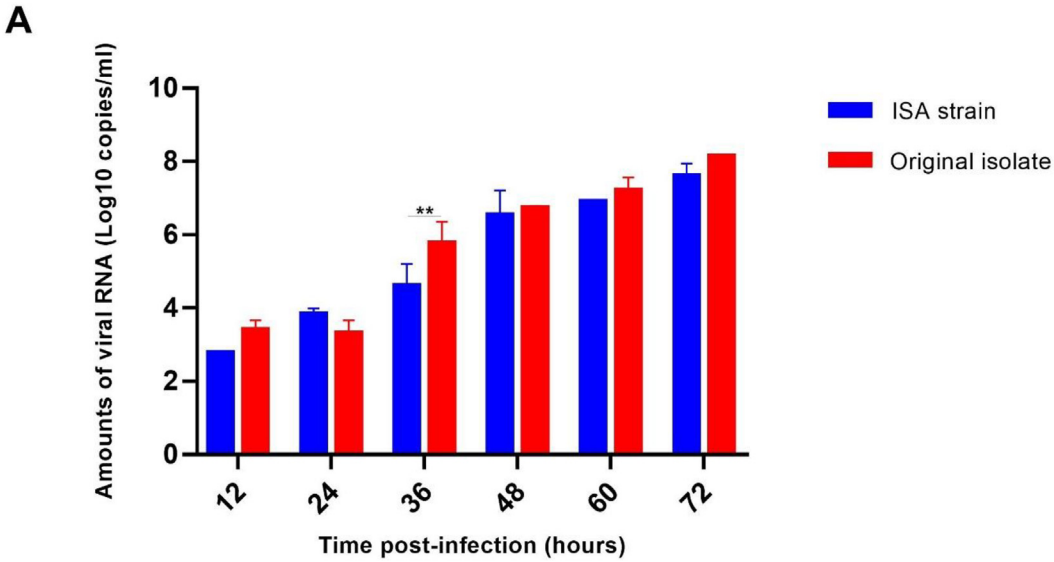
	Cell line Transfection	Passage	CPE	dIFA	Average amount of viral RNA	Infectious titers
Original isolate	–	Marc-Sn	Yes	Yes	6.5 + /- 0.1	6.6 + /- 0.6
ISA strain	Marc-Sn + BHK-21	Marc-Sn	Yes	Yes	7.0 + /- 0.1	5.3 + /- 0.4

promote viruses replicative ability [7,23 12]. In our case, we may hypothesized that the mutations observed may have originated from molecular events that occur during cell culture. GP3, GP4 proteins are known to interact together with GP2 with host cell CD163 [9,48]. It was already established that genetic variations in GP2 and GP4 allow the culture of several PRRRSV strains in Marc-145 cells [51,52]Whilst nsp10 contribute to fatal virulence of HP-PRRSV strains [25] and nsp2 an hypervariable region [42], it appears that genetic variations in those regions are also crucial to promote the viral fitness in cell culture.

The complete genome of the rescued virus was different from 2 consensus mutations in comparison to the *de novo* synthetized fragments. One may supposed that, beyond viral adaptation to cell culture, PCR amplification step during the production of cDNA fragments may enhanced the genetic diversity and improve the chance to rescue infectious viruses [8,34]. However, a recent study on tick-borne encephalitis virus showed that very high-fidelity PCR polymerase during the PCR step of the ISA method may indeed decreased the genetic diversity of the recovered viral population but had no impact of viral fitness and virulence *in vitro* and in animal model [11]. It was also hypothesized that the genetic diversity may not solely depend on the degree of polymerase but also from a viral adaptation that occurred *in vitro*. In our case, we supposed that the use of a higher fidelity polymerase may decreased the generation of quasispecies as described by [11] but still help to rescue infectious still virus's particles (*i.e* limiting fatal error prone PCR amplification) when working with large size viral genomes. Interestingly, when using a lower fidelity polymerase to generate cDNA as used in previous studies [5,4] we failed to recover infectious particles (data not shown).

In conventional PRRSV reverse genetic systems, mutations frequently arise before the rescue process, which can be retained as genetic markers [24], or after the rescue process [44]. The emergence of mutations was already observed in ISA viruses in which genome integrity ranged from 99.95% to 99.99% [3,4,5] consistency with the one observed in this study (99.98%). Thus, if one would carry out a back to back evaluation between recombinant viruses (i.e. recombinant virus with and without desired mutation) it is capital to ensure that wild-type recombinant viruses recapitulates the phenotype of the original isolate. Although it has been reported that mutations in PRRSV genome are responsible for changes in phenotype [1] in our case, the two mutations observed in the rescued virus had no significant effect on viral growth in cell culture.

The CMV promoter aims to initiate DNA fragments transcription using the cells nuclear machinery. In this non-conventional strategy to produce PRRSV, errors may occur during RNA splicing which can impair viral replication particularly for RNA viruses with exclusive cytoplasmic replication such as PRRSV. RNA splicing is a post-transcriptional nuclear mechanism driven by the spliceosome, in which introns are removed from the precursor messenger RNA (pre-mRNA) and exons are ligates resulting in the production of spliced mature mRNA [6,30,40]. However, it has been shown that mutations in the RNA transcript or inside the spliceosome can altered splicing networks (*i.e* generation of newly splicing site,



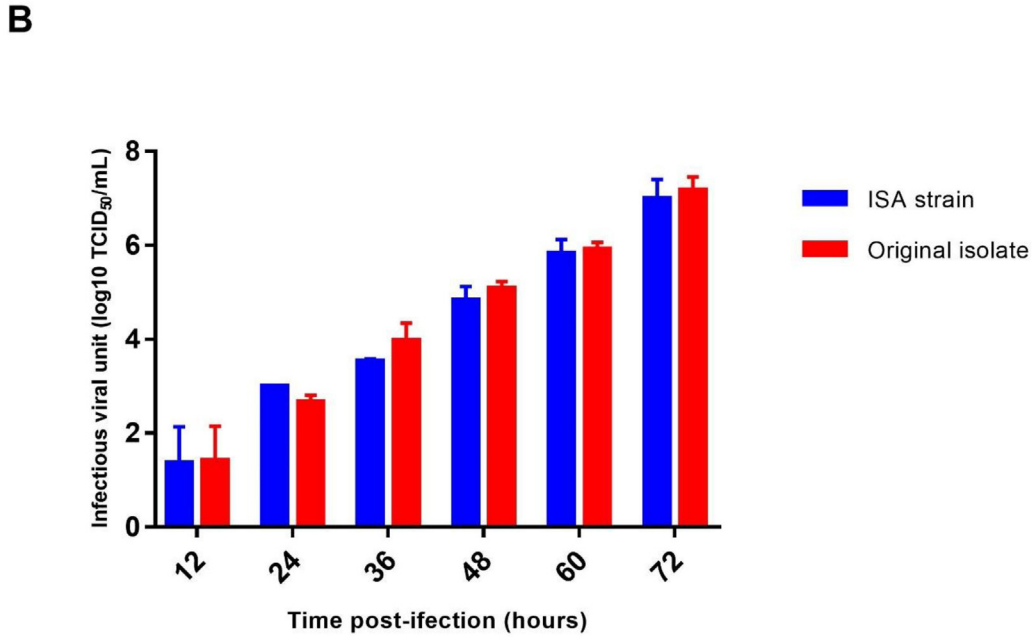


Fig. 5. Virus replication kinetics of original isolate and ISA strain. An MOI of 0.01 was used to infect MARC-Sn cells with PRRSVs original isolate or the ISA strain . Each experiment was performed in triplicate (N = 3). Data are represented as mean ± SD. Exploratory analyses were performed using a two-way ANOVA for multiple comparisons with Sidak's multiple comparisons test. ** : $P < 0.05$.

Table 3
Genotypic characterization. Complete genome analysis of ISA PRRSV strain after 2 passages on MARC-Sn cells using the ISA method. Characteristics of majority mutations and minority mutations are described: position, region, nucleotide and amino acid change, position and frequency of subpopulation observed. Nt: Nucleotide; AA: amino acid;nsp: non-structural protein;"-": silent mutation.

	Nt position	Region	AA position	Nt change	AA change	Frequency (%)
Majority mutation	3458	ORF 1a (nsp2)	1090	C → T	A → V	100
	10230	ORF 1a (nsp10)	882	C → T	–	59
Minority mutation	5753	ORF 1a (nsp4)	1855	C → T		7
	9467	ORF 1ab (nsp9)	627	C → T		9
	10,.248	ORF 1ab (nsp10)	888	A → G		40

exclusion of exons) [16,29]. In order to bypass such events, genomic splicing site predictions and *in silico* codon optimization [47] may be alternatives to removed potential splicing-like sites in DNA fragments and *de facto* promote the viral rescue.

Our approach overcomes traditional and fastidious reverse genetic methods and may be a rapid alternative for rapid next generation of stable recombinant and genetically modified PRRSVs. The utilisation of the ISA approach in the future may help to more

rapidly elucidate new key features of the viral genome. In addition, this method could be used in the future to design attenuated strains of PRRSV.

5. Conclusion

PRRSV is a rapidly evolving and devastating pathogen in swine population. In this study, we *de novo* synthetized and transfected permissive cells with four DNA fragments covering the full-length genome of PRRSV . We successfully reconstructed infectious and wild-type particles of PRRSV with biological characteristics closed to the original isolate. The ISA method represents an asset for the veterinary research community and may facilitate the development of sanitary countermeasures against PRRSV, a challenging pathogen in the swine population.

CRediT authorship contribution statement

Julien Mélade: Conceptualization, Data curation, Formal analysis, Methodology, Investigation, Validation, Visualization, Writing – original draft, Writing - review & editing. **:** . **Géraldine Piorkowski:** Formal analysis, Software. **Hawa Sophia Bouzidi:** Formal analysis. **Alain Medawar:** Funding acquisition, Resources, Project administration. **Claudine Raffy:** Funding acquisition, Resources, Project administration. **Xavier de Lamballerie:** Conceptualization, Funding acquisition, Investigation, Resources, Supervision. **Antoine Nougairède:** Writing – original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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