Baculovirus expression of cloned porcine arterivirus generates infectious particles in both insect and mammalian cells

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ABSTRACT

Studies on several viral pathogens have been hampered by the lack of appropriate *in vitro* systems for their propagation and amplification. Porcine reproductive and respiratory syndrome virus (PRRSV), an arterivirus containing a single-stranded positive-sense RNA genome (∼15 kb), was served as a model virus and its genomic cDNA was recombinated into baculovirus. We investigated whether infectious virus particles could be generated by expression of the full-length cloned genome from the modified baculovirus vector. The recombinant baculovirus, AcAPRRS, was used to infect sf9 cells. Immunofluorescence assay demonstrated the presence of PRRSV nonstructural protein (nsp) 2 and nucleocapsid (N) protein and electron microscopy revealed PRRSV particles in the culture supernatant. Infectious PRRSV particles were also produced in susceptible MARC-145 cells inoculated with AcAPRRS, and the growth characteristics of the PRRSV generated were similar to those of the parental PRRSV strain. Infectious PRRSV particles were also generated following AcAPRRS transduction of BHK-21 cells and Vero cells that are not sensitive to PRRSV. Titers of PRRSV obtained from BHK-21 and Vero cells were up to $10^{4.05}$ TCID$_{50}$/ml. These findings open a new route to the propagation of the virus *in vitro* and will be of utility in vaccine development.

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1. Introduction

Many viral pathogens including Norwalk virus and hepatitis C virus (HCV), with the exception of strain JFH-1, have as yet no *in vitro* cell culture system (Asanaka et al., 2005; Duverlie and Wychowski, 2007). The absence of an appropriate *in vitro* system for their propagation and amplification has constrained studies on such viruses and has hindered vaccine development. Attempts have been made to employ fowlpox virus, adenovirus and baculovirus as delivery vectors for expression of hepatitis B virus (HBV) genomic DNA or HCV genomic cDNA in hepatocytes, and infectious virus particles were reported to be produced in the transduced cells (Lucifora et al., 2008; Ren and Nassal, 2001; Yao et al., 2008; Yap et al., 1998).

The insect baculovirus is a versatile tool that has been applied to the production of high levels of recombinant proteins, the display of foreign peptides on virus particles, and the gene delivery in mammalian cells and *in vivo* (Kost et al., 2005; Tani et al., 2003). In addition, recombinant baculovirus has been used to amplify an adeno-associated virus vector in insect cells and recently *Rhopalosiphum padi* virus (RhPV), an insect virus that infects aphids, was successfully generated in insect cells from recombinant baculovirus

(Aslanidi et al., 2009; Pal et al., 2007). However, it remains an open question whether vertebrate viruses can be generated in insect cells by use of appropriate baculovirus recombinants. In this paper, we have used porcine reproductive and respiratory syndrome virus (PRRSV) as a model virus to address whether infectious mammalian virus particles can be generated in insect cells from recombinant baculovirus.

PRRSV is a small enveloped virus belonging to the family *Arteriviridae*, order *Nidovirales*. The PRRSV genome, a single-stranded positive-sense RNA approximately 15 kb in length with a 5′ cap and a 3′ polyA-tail, contains at least nine open reading frames (ORFs). ORFs 1a and 1b encode a polyprotein that is processed co- and post-translationally to generate the mature nonstructural protein (nsp) required for virus replication. The other seven ORFs encode seven structural proteins designed glycoprotein (GP) 2, envelope (E) protein, GP3, GP4, GP5, membrane (M) protein and nucleocapsid (N) protein, respectively (Snijder and Meulenberg, 1998). Expression of PRRSV structural proteins, as reported for coronavirus structural proteins, requires subgenomic (sg) mRNA synthesis by a mechanism of discontinuous transcription (Pasternak et al., 2006). The only known natural host for PRRSV is the pig, and the virus has been shown to infect primary porcine alveolar macrophages (PAM) as well as a monkey kidney epithelial cell line, MA-104, and its derivatives including MARC-145 cells.

In mainland China, PRRS is the most economically important disease of swine herds. A new PRRS outbreak was first described in

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Table 1
Primers used for PCR and RT-PCR.

Primer name	Sequence (5′–3′)[a]	Location[b]
CMV-SF1	ACCGCATGCACGCGTAGGTATGACGTATAGGTGTTGGC	1–19 nt
SF12	GTGTTGGCTCTATGCCTTGAC	12–32 nt
SR683	GAGCGGCAGGTTGGTTAA	665–682 nt
SR1124	CTTGCAGCCTCCGCTGTAGGTA	1103–1124 nt
SR2573	CTGCCCAGGCCATCATGTCCGAAGTC	2548–2573 nt
SF14413	CTGATCGACCTCAAAAGAGTTGTGCTTG	14,413–14,440 nt
SR15497	CAATTAAATCTTACCCCCACACGGTCG	15,471–15,497 nt
Qst	TTTTTTTTTTTTTTTTTTT	Poly(A)
PCMVF	GGTGGCGGCCGCTAGTTATTAATAGT	CMV promoter
PCMVR	CGTGCATGCACCTCTGCTTATATAG	CMV promoter
PHDVR1	CGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTTTTAATTTCGGCCGCATGGTTTTC	Hepatitis delta virus ribozyme
PHDVR2	GAGTGGACGTGCGTCCTCCTTCGGATGCCCAGGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCC	Hepatitis delta virus ribozyme
PHDVR3	TGCCTCGAGCGCCCTCCCTTAGCCATCCGAGTGGACGTGCGTCCTCCTTC	Hepatitis delta virus ribozyme

^a Restriction sites are underlined.
^b The position of viral genomic RNA (GenBank no. GQ330474).

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[b] The position of viral genomic RNA (GenBank no. GQ330474).

mid-2006, and 'porcine high fever disease' (PHFD) subsequently spread throughout the Chinese swine industry, resulting in the culling of an estimated 30 million pigs. A highly virulent PRRSV variant has been confirmed to cause PHFD (Lv et al., 2008; Tian et al., 2007). Several different types of inactivated or modified live vaccines have been trialed against PHFD but have only met with limited success, and a major research focus has been to develop a safe and effective vaccine against the new variant.

In the present study, we explored the use of a recombinant baculovirus vector to direct the expression of the full-length PRRSV genome under the dual control of CMV and polyhedrin promoter sequences. The recombinant virus was used to infect insect or transduce mammalian cells. We report that infectious PRRSV particles can be generated after either infection of sf9 insect cells or transduction of mammalian cells. These findings suggested a new strategy for virus amplification *in vitro* and vaccine development.

2. Materials and methods

2.1. Virus and cells

Spodoptera frugiperda sf9 cells were cultured at 27 °C in Sf-900 serum-free medium (Invitrogen). MARC-145 cells were cultured at 37 °C under 5% CO_2 in Eagle's minimal essential medium (JRH) supplemented with 10% fetal bovine serum (FBS). BHK-21 cells and Vero cells were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. The PRRSV strain APRRS (GenBank Accession no. GQ330474) and its infectious clone pAPPRS were prepared and maintained as described previously (Yuan and Wei, 2008). Recombinant baculovirus AcLacZ was generated from sf9 cells transfected with the Bacmid isolated from *Escherichia coli* DH10Bac^{TM} and was used as a control virus.

2.2. Construction of DNA-based PRRSV recombinant virus

The CMV promoter segment was amplified from pCMV-Script (Stratagene) with the primer pair PCMVF and PCMVR (Table 1) and PfuUltra® Hotstart DNA Polymerase (Stratagene). The amplified segment was cleaved with *Not*I and ligated into *Not*I- and *Eco*RI-cleaved pBluescript SK(+) to generate recombinant plasmid pBCMV.

A 5′-segment of the PRRSV genome was amplified from pAPRRS, using PfuUltra® Hotstart DNA polymerase and primers CMV-SF1/SR2573 (Table 1), and digested with *Sph*I before cloning. Plasmid pBCMV was digested with *Sal*I, filled in using T4 polymerase, and cleaved with *Sph*I before insertion of the 5′-end segment by ligase treatment, generating plasmid pC-ARS.

Plasmids pC-ARS and pAPRRS were cleaved with *Not*I plus *Afl*II and religated to substitute the T7 promoter sequences in pAPRRS by CMV promoter sequences, generating plasmid pCAPRRS (Fig. 1). Hepatitis delta virus (HDV) antigenomic ribozyme sequences were PCR amplified with primers SF14413 and PHDVR1, PHDVR2, PHDVR3, and *Xba*I and *Xho*I cleavage and religation was used to insert the segment containing HDVr into pCAPRRS.

2.3. Construction of a recombinant baculovirus carrying PRRSV genomic cDNA

A recombinant baculovirus containing the genomic cDNA of APRRS, designated AcAPRRS, was generated using the Bac-to-Bac Baculovirus Expression System (Invitrogen). First, the full-length cDNA in clone pCAPRRS was excised with *Not*I plus *Xho*I, and ligated into pFastbac1 digested with the same enzymes. The recombinant donor plasmid, pBacAPS, was identified by *Sma*I digestion and transformed into *E. coli* DH10Bac^{TM}. Recombinant Bacmids were selected following manufacture's instruction, and identified by PCR using of the primer pairs SF12/SR683 and SF14413/SR15497 (Table 1). The recombinant baculovirus was generated following manufacture's instruction. Briefly, sf9 cells were grown to 70% confluence in 35 mm culture dishes and transfected with the recombinant Bacmid using Cellfectin (Invitrogen). 5 days after transfection, cell supernatants were harvested. Recombinant baculovirus was confirmed by PCR with two pairs of primers, SF12/SR683 and SF14413/SR15497 (Table 1). This preparation was used to infect sf9 cells, generating a virus stock. This stock was stored as aliquots at −70 °C.

2.4. Purification of PRRSV particles from sf9 cells

Sf9 cells (1×10^7) in T-75 flasks were inoculated with AcAPRRS at a multiplicity of infection (MOI) of 0.2. After incubation for 1 h at 27 °C, 40 ml of fresh medium was added to the flasks and incubation continued for a further 7 days at 27 °C. Supernatants were clarified by centrifugation in two steps, first at $376 \times g$ for 20 min and then at $9400 \times g$ for 30 min (Sigma, H-12150). To remove AcAPRRS, supernatants were centrifuged twice at $50,000 \times g$ for 1 h at 4 °C (Beckman, SW32Ti). PRRSV particles were then precipitated by centrifugation through a 30% sucrose cushion at $106,750 \times g$ for 2 h (BeckMan, SW32 Ti). The pellet was resuspended in STE buffer. A drop of the final suspension or AcAPRRS suspension was placed on a formvar–carbon-coated copper grid, negatively stained with 1% phosphotungstic acid for 1 min, then observed under the electron microscope (JEM2100).
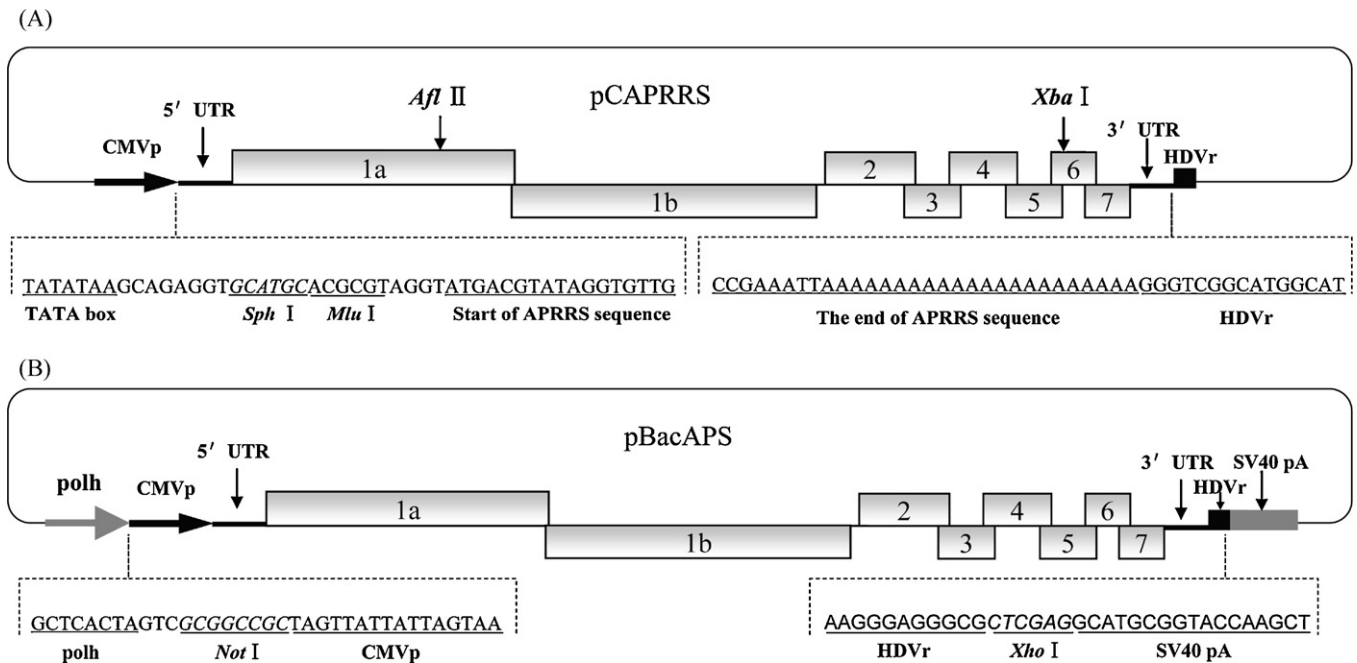
(A)

(B)

Fig. 1. Schematic maps of vectors used for recombinant baculovirus construction. (A) Structure of pCAPRRS. CMVp, CMV promoter; HDVr, hepatitis delta virus (HDV) ribozyme. The sequences between CMVp and HDVr comprise the intact genomic cDNA of PRRSV APRRS; the genome organization of PRRSV is indicated. The vector was pBluescript SK(+). (B) Structure of donor plasmid pBacAPS. Polh, polyhedrin promoter; CMVp, CMV promoter; HDVr, HDV ribozyme; SV40 pA, SV40 polyadenylation signal. The genomic cDNA of PRRSV under CMVp control was inserted downstream of polh. The vector was pFastbac1.

2.5. Detection of PRRSV RNA

To detect PRRSV genomic RNA, the final suspension as described above were used to isolate PRRSV RNA with RNAprep Pure Cell/Bacteria Kit (TIANGEN) according to the manufacturer's instructions. DNaseI was used to eliminate contaminating DNA. Reverse transcription (RT) employed the primer Qst and SR2573, and was followed by PCR using the primer pairs SF14413/SR15497 and SF12/SR683.

2.6. Immunofluorescence assay (IFA)

Aliquots of sf9 cells (8×10^5/dish) in 35 mm plates were inoculated with AcAPRRS or AcLacZ at an MOI of 0.2, or with PRRSV APRRS at an MOI of 5, respectively. After incubation for 1 h at 27 °C, cells were washed once with Sf-900 serum-free medium, 3 ml fresh medium was added to each dish, and incubation was continued for 4 days at 27 °C. Cell supernatants were discarded and the cell layer was washed once with phosphate-buffered saline (PBS) before IFA.

MARC-145 cells (5×10^5/well) in 6-well plates were inoculated with AcAPRRS or AcLacZ at an MOI of 5, or with PRRSV APRRS at an MOI of 0.005, respectively. After 2 h incubation at 37 °C, cells were washed twice with PBS before adding fresh medium (MEM plus 2% FBS). Cells were incubated at 37 °C for a further 3 days and washed once with PBS before IFA.

BHK-21 cells and Vero cells (5×10^5/well) in 6-well plates were inoculated with AcAPRRS, AcLacZ, or PRRSV APRRS at an MOI of 5 and incubated for 8 h at 37 °C. Cells were washed twice with PBS before adding fresh medium (DMEM plus 2% FBS). After 3 days, cells were washed once with PBS before IFA.

For IFA, cell layers were fixed by adding ice-cold anhydrous methanol and incubating for 10 min at −20 °C. Cells were then permeabilized with 0.1% tween-20 in PBS at room temperature for 5 min after blocking with 2% BSA in PBS for 30 min at 37 °C, the cells incubated for 1.5 h at 37 °C with anti-PRRSV N protein monoclonal antibody (mAb), anti-PRRSV nsp2 mAb (kindly provided by Dr. Ying Fang at South Dakota State University), or anti-AcMNPV

GP64 protein mAb (AcV1, Santa Cruz) at 1:800 dilution in PBS plus 1% BSA. After washing, bound antibody was detected with a goat anti-mouse IgG conjugated to Alexa Fluor 586 (Invitrogen).

2.7. Virus titration and plaque assay

PRRSV titers were determined as $TCID_{50}$/ml on MARC-145 cells. Serial 10-fold dilutions of virus were prepared in MEM and transferred to 96-well plates (50 μl/well). 100 μl aliquots of MARC-145 cells (3×10^5 cells/ml) were added to each well and the plates were incubated for 7 days at 37 °C under 5% CO_2. Titers in $TCID_{50}$/ml were calculated from cytopathic effects (CPE) according to the Reed–Muench formula.

The virus vASM, *in vitro* generated from MARC-145 cells inoculated with AcAPRRS, or PRRSV APRRS was serially 10-fold diluted. 0.2 ml viruses were inoculated onto MARC-145 monolayers in 6-well plates. After 1 h at 37 °C, cell monolayers were washed once with PBS and overlaid with 3 ml MEM containing 1% (w/v) low melting-point agarose and 2% FBS. After incubation for a further 4 days at 37 °C, plaques were stained using 5% (w/v) crystal violet.

2.8. 5'- and 3'-end sequences of vASM

To determine the sequences of 5'- and 3'-ends of vASM and its passage on MARC-145 cells, the 5'-Full Race Kit and 3'-Full Race Core Set (Takara) were used according to the manufacturer's protocols. Extraction of total RNA employed the QIAamp Viral RNA Mini Kit. For 5'-RACE, primer SR2573 was used for the RT step and the primers SR1124 and SR683 (Table 1) were used for nested PCR in combination with the universal primers provided with the kit. For 3'-RACE, primer SF14413 and the primer provided with the kit were employed. PCR products were purified using a Gel Extraction Kit (WATSON Inc.) and cloned into vector pGEM-T for sequencing.
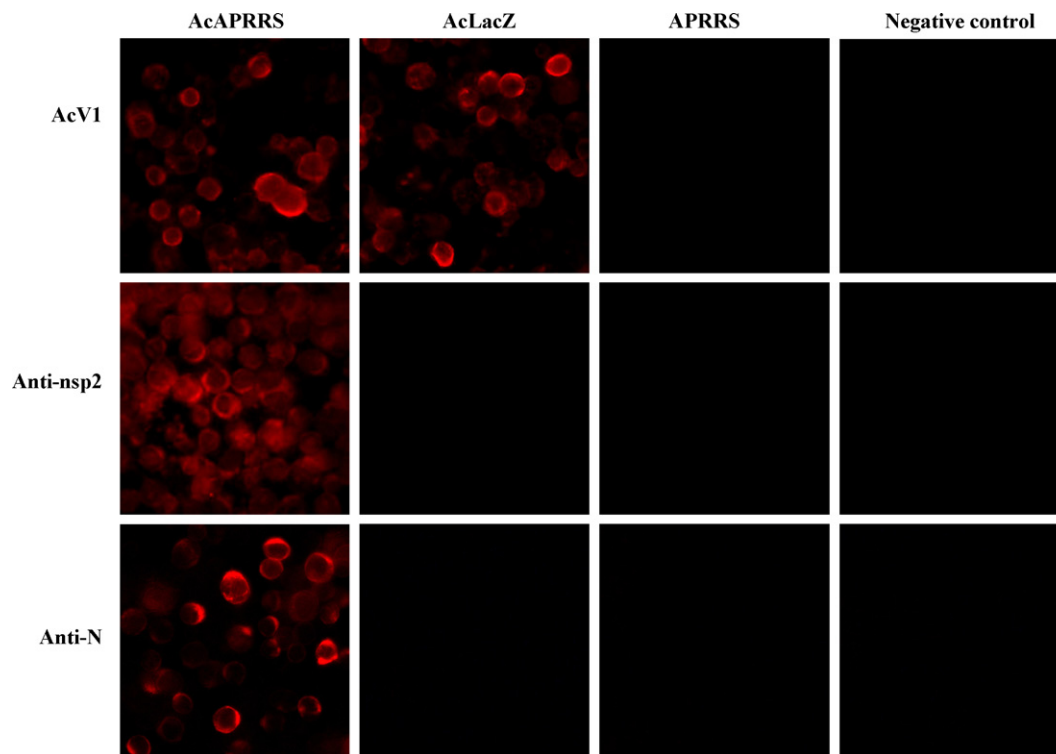
Fig. 2. PRRSV proteins expressed from recombinant baculovirus-infected sf9 cells. Immunofluorescence assay (IFA) using mAbs specific for nsp2 and N proteins of PRRSV or GP64 of AcMNPV. Cultures of sf9 were inoculated with recombinants AcAPRRS or AcLacZ, or with virus PRRSV APRRS, and IFA was performed 4 days post-infection. Only the cells infected with AcAPRRS showed positive staining for the PRRSV proteins.

2.9. Kinetics of PRRSV generated from BHK-21 and Vero cells transduced with AcAPRRSV

BHK-21 cells and Vero cells (5×10^5/dish) were cultured in 35 mm dishes and inoculated with AcAPRRS at an MOI of 5. After incubation at 37 °C for 8 h, cells were washed twice with PBS and 3 ml of DMEM plus 2% FBS was added to each dish. At serial 8 h post-infection timepoints 200 μl supernatant samples were collected for evaluation and replaced by 200 μl of medium. Viral titers were determined by the $TCID_{50}$/ml on MARC-145 cells. Time-curves of PRRSV titers were drawn using GraphPad Prism 4 software.

3. Results

3.1. Generation of a recombinant baculovirus, AcAPRRS, containing full-length PRRSV genomic cDNA

Genomic clone pAPRRS was modified to produce a virus capable of generating PRRSV on transfection of mammalian cells. First, the T7 promoter sequence was replaced by the major cytomegalovirus (CMV) promoter. Second, the self-cleaving hepatitis delta virus ribozyme (HDVr) sequence was inserted at the 3′-end of the PRRSV genomic cDNA (Fig. 1A). The donor plasmid, pBacAPS (Fig. 1B), was constructed to allow transfer to the baculovirus genome. Then, a recombinant baculovirus, AcAPRRS, was generated using the bac-to-bac system. Recombinant baculovirus AcAPRRS contained a complete PRRSV genomic cDNA 735 bp downstream of the polyhedrin promoter and immediately downstream of the CMV promoter. At the 3′ the genomic cDNA was followed by the self-cleaving HDVr sequence and a SV40 polyadenylation site.

3.2. PRRSV genes are expressed in sf9 cells

To determine whether the PRRSV genome contained within AcAPRRS was expressed in insect cells, sf9 cells were infected with AcAPRRS, and immunofluorescence was performed 4 days post-infection using mAb specific for PRRSV proteins N and nsp2. Sf9 cells infected with AcAPRRS displayed positive immunofluorescence for both N and nsp2, whereas the sf9 cells inoculated with PRRSV APRRS and AcLacZ were negative (Fig. 2). These results demonstrated that native PRRSV was unable to infect sf9 cells, but the PRRSV genome of AcAPRRS was transcribed and translated to generate PRRSV-specific proteins. The production of PRRSV structural proteins requires expression of subgenomic mRNA (Snijder and Meulenberg, 1998). This also demonstrated that the PRRSV genomic cDNA carried by AcAPRRS was subject to genomic RNA transcription, and then subgenomic mRNA was discontinuously transcribed.

3.3. Generation of PRRSV particles in sf9 cells

To investigate whether PRRSV particles were generated following infection of insect cells with the recombinant baculovirus, sf9 cells were infected with AcAPRRS and supernatants were harvested 7 days later. The parental AcAPRRS virus was first removed by centrifugation at $50,000 \times g$. Virus particles still present in the supernatant were collected by high-speed centrifugation ($106,750 \times g$) and examined by electron microscopy. As shown in Fig. 3A, the final supernatants contained virus particles 45–60 nm in diameter that were morphologically similar to PRRSV virions. However, the particles of AcAPRRS were morphologically identical to baculovirus virions (Fig. 3B). To address whether these particles in final supernatants contained PRRSV genomic RNA, the preparations were digested with DNaseI and then subjected to RT-PCR using PRRSV-specific primers. As shown in Fig. 3C and D, omission of the RT step failed to generate any PCR product (lane 3), whereas RT-PCR generated a PCR product of the expected size (lane 2). Because PCR amplification of PRRSV sequences was crucially dependent on RT, we concluded that virions containing PRRSV RNA were generated in sf9 cells.
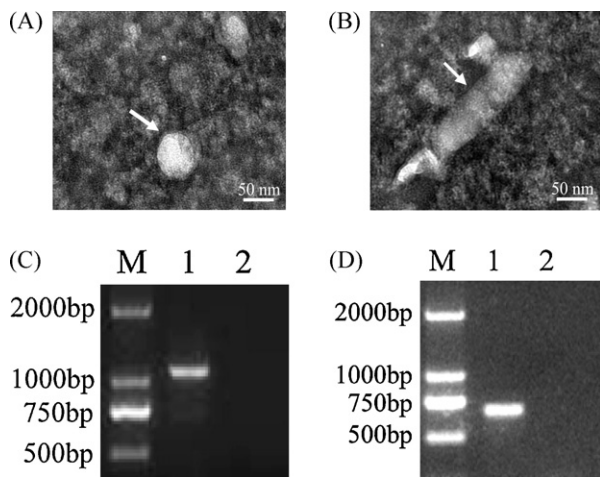
Fig. 3. Virus particles in the supernatants of sf9 cells infected with AcAPRRS. (A) PRRSV particles observed by electron microscopy. Residual AcAPRRS particles were removed by centrifugation ($50,000 \times g$) and PRRSV virions (45–60 nm; arrowed) were collected by high-speed centrifugation ($106,750 \times g$) and visualized after negative staining. (B) AcAPRRS particles observed by electron microscopy. The particles of AcAPRRS were precipitated by centrifugation ($50,000 \times g$) and resuspended in STE. AcAPRRS particles (arrowed) were negatively stained and observed under the electron microscope. (C) PCR amplification of PRRSV sequences with primer pairs SF14413/SR15497. Clarified supernatants were examined following ultracentrifugation ($106,750 \times g$) and DNaseI treatment. PCR with primer pairs SF14413/SR15497 was then performed either with or without a RT step with the primer Qst as indicated. The signal in lane 1 was insensitive to prior DNaseI treatment, but was abolished when the RT step was omitted (lane 2). Lane M, DNA marker mixture DL2000. (D) PCR amplification of PRRSV sequences with primer pairs SF12/SR683. After the preparations digested with DNaseI, PCR with primer pairs SF12/SR683 was then performed either with or without a RT step with the primer SR2573. The signal in lane 1 was insensitive to prior DNaseI treatment, but was abolished when the RT step was omitted (lane 2). Lane M, DNA marker mixture DL2000.

3.4. PRRSV production in MARC-145 cells

Cultured MARC-145 cells were inoculated with AcAPRRS; control cultures were inoculated with either AcLacZ or PRRSV APRRS. Cells were examined by immunofluorescence at 3 days post-inoculation. As shown in Fig. 4A, cells inoculated with either AcAPRRSV or PRRSV APRRS showed positive staining with mAb directed against PRRSV N protein; cells inoculated with AcLacZ were negative. The subcellular localization of N protein from AcAPRRS was indistinguishable from that produced from PRRSV APRRS.

These results indicated that MARC-145 cells inoculated with AcAPRRS might generate infectious PRRSV virions. To address this possibility, we examined CPE on the MARC-145 cells after inoculation of the AcAPRRS and control viruses. This revealed pronounced CPE of MARC-145 cells inoculated with AcAPRRS (MOI = 5.0) at 3 days post-infection, whereas no CPE were detectable in cells inoculated in parallel with AcLacZ at the same MOI. The supernatant of the MARC-145 cells inoculated with AcAPRRS, designated vASM, was diluted 1:100 and reinoculated onto fresh MARC-145 cells. As a control, the undiluted supernatant of the MARC-145 cells inoculated with AcLacZ was also inoculated onto fresh cells. Inspection of the plates revealed strong CPE at 2 days post-infection in the cells inoculated with the vASM preparation, whereas control cells and cells infected with the AcLacZ supernatants showed no CPE even at 7 days post-inoculation. The presence of PRRSV sequences in the reinoculated cells was investigated using RT-PCR. This revealed that both the first and second passages of the MARC-145 supernatants (vASM) were positive for PRRSV sequences, whereas parallel cultures of AcLacZ were systematically negative (Fig. 4B). These results argued that infectious PRRSV particles were produced following MARC-145 cells inoculated with AcAPRRS.

To confirm that virions produced in insect culture were indeed infectious to mammalian cells, Sf9 cells were infected with AcAPRRS (MOI = 0.2) and supernatant samples were taken at different timepoints, indicated in Fig. 4C. These were titrated by serial dilution, inoculation onto MARC-145 cells, and examination for PRRSV production. As shown in Fig. 4C, sf9 culture supernatant harvested at 48 h post-infection contained PRRSV at $10^{1.2}$ TCID$_{50}$/ml, and the peak titer ($10^{3.3}$ TCID$_{50}$/ml) was achieved by the 6-day post-inoculation samples (Fig. 4C).

3.5. Characteristics of baculovirus-derived PRRSV

We then compared the growth properties of newly generated PRRSV with those of the parental PRRSV APRRS strain. MARC-145 cells were infected with either the vASM preparation or with APRRS; culture supernatants were titered for infectious particles at different timepoints post-infection. As shown in Fig. 5A, the growth kinetics of vASM were similar to those of APRRS. Although vASM grew slightly slower than APRRS, the peak titers of the two preparations were similar. In addition, the plaque sizes of vASM and APRRS on MARC-145 cells were not significantly different (Fig. 5B). These findings argued that the growth characteristics of the baculovirus-derived vASM were highly similar to those of the parental PRRSV APRRS virus. The results of 5′- and 3′-RACE showed that the terminal sequences of both the first and second passages of vASM were identical to those of PRRSV APRRS (data not presented).

3.6. PRRSV generation in BHK-21 and Vero cells

MARC-145 cells have previously been shown to be susceptible to infection by PRRSV, and we therefore studied whether other mammalian cell lines can express PRRSV antigens and/or generate virus particles. First, BHK-21 and Vero cells were inoculated with AcAPRRS; immunofluorescence analysis was performed using mAb specific for PRRSV N protein. As shown in Fig. 6A, both cell lines were positive for PRRSV fluorescence, whereas controls cultures infected with either PRRSV APRRS or AcLacZ were negative. These results demonstrated that both BHK-21 and Vero cell lines were refractory to infection with PRRSV, but moreover that the recombinant baculovirus carrying PRRSV genomic cDNA can transduce these cells and express the PRRSV genome.

We then examined whether these cells can also generate infectious PRRSV particles. When day 3 post-inoculation supernatants were inoculated onto MARC-145 cells, CPE were detected as early as 2 days post-inoculation, whereas no CPE were detected following inoculation from control AcLacZ-infected cultures even after 7 days of incubation. We used RT-PCR to confirm that the supernatants of the MARC-145 cells were positive for PRRSV sequences whereas AcLacZ supernatants were negative (Fig. 6B). These results indicated that the transduction of either BHK-21 or Vero cells with AcAPRRS led to the generation of infectious PRRSV particles.

To address the kinetics of PRRSV virion production in the BHK-21 and Vero lines, cells were inoculated with AcAPRRS (MOI = 5.0) and cell supernatants were titered at different timepoints. As shown in Fig. 6C, infectious PRRSV particles were generated somewhat faster on BHK-21 cells than on Vero cells, but the peak PRRSV titer on Vero cells ($10^{4.28}$ TCID$_{50}$/ml) was slightly higher than that obtained with BHK-21 cell ($10^{4.05}$ TCID$_{50}$/ml). The finding that PRRSV titers rose markedly following inoculation further argues that PRRSV virions were actively generated from BHK-21 cells and Vero cells transduced with AcAPRRS.
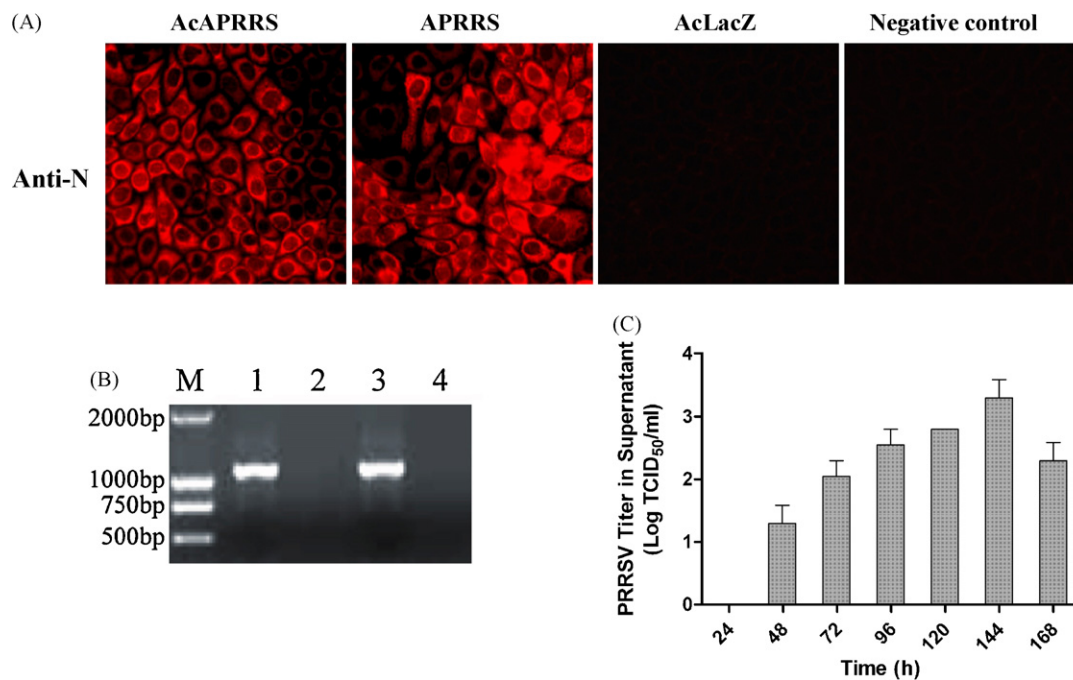
Fig. 4. Infectious PRRSV produced in MARC-145 cells. (A) Immunofluorescence of 4-day post-infection MARC-145 cells infected with different recombinants using mAb directed against PRRSV N protein. Both AcAPRRS- and PRRSV–APRRS-inoculated cells demonstrated specific staining whereas cells inoculated with AcLacZ and control cells were negative. (B) RT-PCR analysis of supernatants of recombinant baculovirus-inoculated MARC-145 cells using PRRSV-specific primers. Lane 1 and lane 3, first and second passages of virus on MARC-145 cells, respectively; lane 2 and lane 4, first and second passages of control virus AcLacZ on MARC-145 cells, respectively. Only the supernatants from AcAPRRS-inoculated cells were positive for PRRSV sequences. (C) Evolution of virus titers in cultures of insect cells inoculated with AcAPRRS. Virus was titrated by serial dilution and the detection of cytopathic effects on MARC-145 cells at 7 days post-infection.

4. Discussion

The baculovirus *Autographa californica* multicapsid nucleopoly-hedrovirus (AcMNPV) is a large double-stranded DNA virus with a circular genome of about 134 kb in length (Ayres et al., 1994). In view of their large genome size and flexibility, baculoviruses can accommodate insertion of large segments of foreign DNA (Hu, 2005). We reported the construction of a recombinant baculovirus, AcAPRRS, containing a fragment of ~16 kb comprising the full-length genomic cDNA of PRRSV under the control of the major CMV promoter.

Baculovirus vectors have previously been exploited to amplify viruses that propagate on hepatocytes, including HBV and HCV, but the baculovirus approach has not yet been applied to the amplification of viruses that lack appropriate cell lines for *in vitro* propagation (Lucifora et al., 2008; Yao et al., 2008). We report that infection of insect sf9 cells with the recombinant baculovirus AcAPRRS led to the synthesis of PRRSV proteins and the culture supernatant contained particles that were morphologically indistinguishable from PRRSV virions. These findings demonstrated that the PRRSV genomic cDNA contained within AcAPRRS was transcribed for protein synthesis and PRRSV RNA was actively packaged into virus particles. The recombinant baculovirus approach could therefore provide a general method for propagating viruses, notably RNA viruses, for which no appropriate *in vitro* culture system is as yet available.

In the present study, the titers of PRRSV particles produced from baculovirus-infected insect cells were relatively modest ($\sim 10^{3.3}$ TCID$_{50}$/ml). This may be due to the fact that the genomic cDNA is inserted downstream of two promoters, the polyhedrin promoter and the CMV promoter. Transcription initiation at the CMV promoter was thought to generate intact genomic PRRSV RNA, but the CMV promoter is only poorly active in insect cells (He et al., 2008). Further experiments will be required to address whether direct linkage of PRRSV genomic sequences to a strong promoter
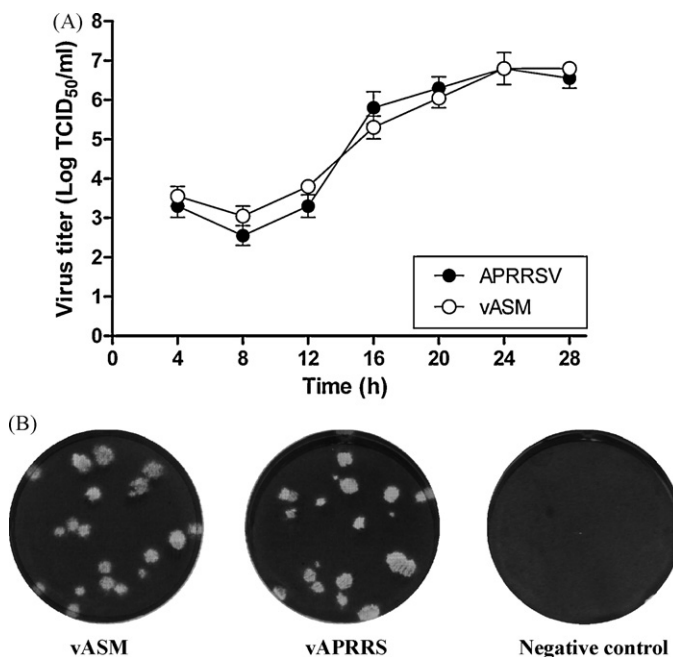

Fig. 5. One step growth curve and plaque morphology. (A) One step growth curve of vASM, the supernatants of MARC-145 cells inoculated with the recombinant baculovirus AcAPRRS, and APRRS on MARC-145 cells. MARC-145 cells were infected with vASM and APRRS at 5 MOI. The supernatant samples were taken at different timepoints and viruses were titered by serial dilution and plating on MARC-145 cells. (B) Plaque morphology of vASM and APRRS on MARC-145 cells. Infected cell monolayers (4 days post-infection) were stained with crystal violet; plaque morphologies were indistinguishable.
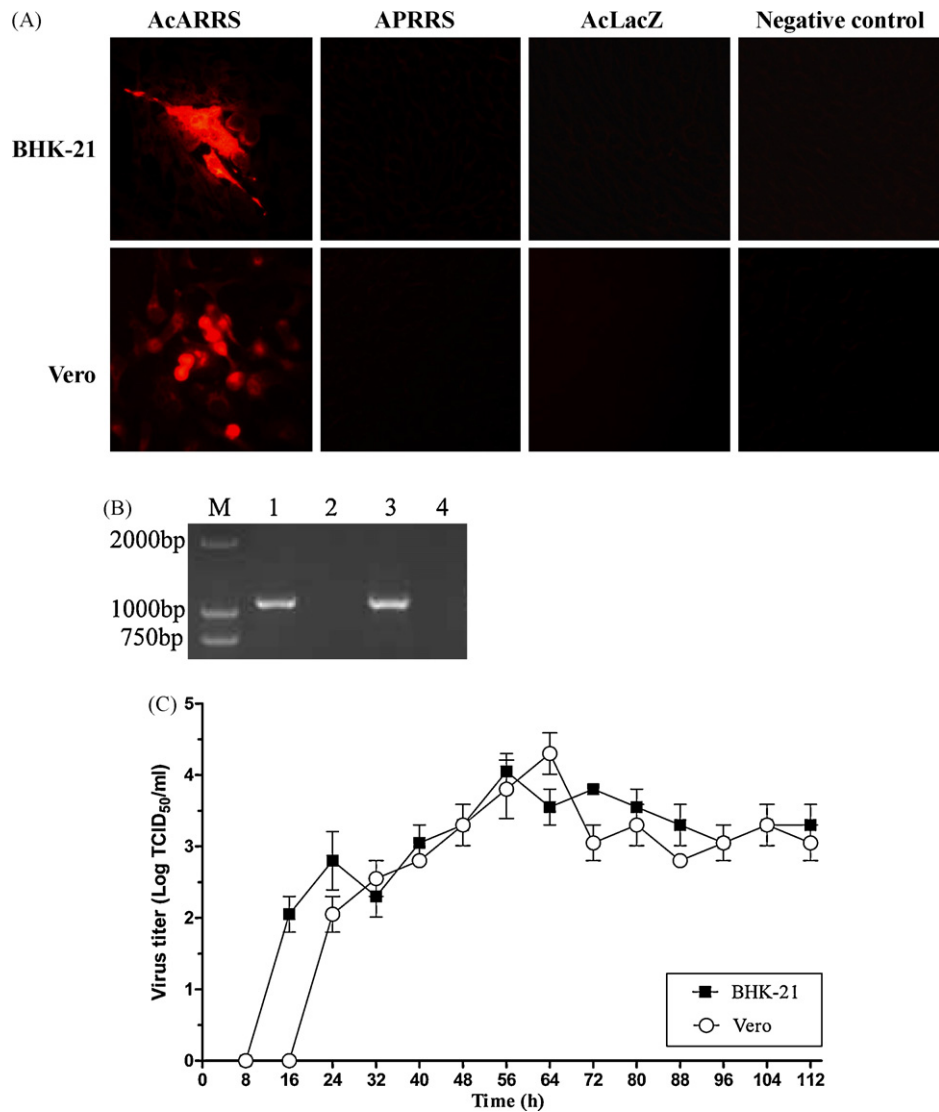
Fig. 6. Infectious PRRSV generated in BHK-21 and Vero cells. (A) Immunofluorescence using mAb directed against PRRSV N protein. AcAPRRS, AcLacZ or PRRSV APRRS was separately inoculated onto BHK-21 cells and Vero cells, immunofluorescence was performed at 3 days post-inoculation. Only cells inoculated with AcAPRRS showed positive staining. (B) RT-PCR analysis of cultures of MARC-145 cells infected with supernatants of recombinant baculovirus-inoculated BHK-21 or Vero cells using PRRSV-specific primers. Lane 1, the cultures of MARC-145 cells infected with the supernatant of AcAPRRS-inoculated BHK-21; lane 2, the cultures of MARC-145 cells infected with the supernatant of AcLacZ-inoculated BHK-21 cells; lane 3, the cultures of MARC-145 cells infected with the supernatant of AcAPRRS-inoculated Vero cells; lane 4, the cultures of MARC-145 cells infected with the supernatant of AcLacZ-inoculated Vero cells. Only the cultures from AcAPRRS-inoculated cells were positive for PRRSV sequences. (C) Growth curve of PRRSV generated in BHK-21 and Vero cells as indicated; titers were determined at the specified timepoints by serial dilution and plating on MARC-145 cells.

sequence active in insect cells would significantly increase virus titers.

In our study, when the recombinant baculovirus AcAPRRS was inoculated onto BHK-21 or Vero cells, PRRSV titers of $10^{4.05}$ TCID$_{50}$/ml or $10^{4.28}$ TCID$_{50}$/ml were obtained, respectively. Baculovirus is generally considered to be a safe vector. Transduction of mammalian cells appears to take place efficiently but there are no manifest adverse effects and the baculovirus genome is not transcribed and is unable to replicate (Kost and Condreay, 2002; Tjia et al., 1983). Baculovirus can transduce diverse mammalian cell types and has been exploited as a vector for gene therapy and for DNA vaccine delivery (Kost and Condreay, 2002; Tani et al., 2003). It was previously reported that baculovirus expressing the E2 glycoprotein of HCV under the control of the CMV immediate-early promoter–enhancer elicited specific humoral and cell-mediated immune responses following intramuscular inoculation of mice (Facciabene et al., 2004). In this study, we have demonstrated that a recombinant baculovirus carrying PRRSV genomic cDNA was able to generate infectious PRRSV following transduction of mammalian cells that are themselves refractory to infection with PRRSV in vitro. This approach may therefore provide an alternative route for vaccine development, particularly if combined with the expression of genomic cDNA derived from an attenuated virus strain. It is possible that direct inoculation of such a recombinant baculovirus into animals or humans could generate infectious vaccine virus in vivo and thereby induce effective anti-viral immunity. Because baculovirus can be cultured on insect cells in the absence of serum, this avoids the potential risks of contamination associated with the use of animal products.

In conclusion, we have constructed a recombinant baculovirus carrying PRRSV genomic cDNA downstream of two promoters: the polyhedrin promoter active in insect cells and a CMV promoter active in mammalian cells. When the recombinant baculovirus was inoculated onto insect sf9 cells or mammalian Vero and BHK-21 cells, infectious PRRSV virions were generated. The approach described here provides a new baculovirus-based strategy for vaccine development and the production of viruses for which no appropriate in vitro culture system is as yet available.

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