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Establishment of Bi-segmented and Tri-segmented Reverse Genetics Systems to Generate Recombinant Pichinde Viruses

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Abstract

Pichinde virus (PICV), isolated from rice rats in Colombia, South America, is an enveloped arenavirus with a bi-segmented RNA genome. The large (L) genomic segment encodes the Z matrix protein and the L RNA-dependent RNA polymerase, whereas the small (S) genomic segment encodes the nucleoprotein (NP) and the glycoprotein (GPC). This article describes the successful development of reverse genetics systems to generate recombinant PICV with either a bi-segmented or tri-segmented genome. We have successfully demonstrated that these systems can generate high-titered and genetically-stable replication-competent viruses from plasmid transfection into appropriate cell lines. These systems demonstrate the power and versatility of reverse genetic technology to generate recombinant arenaviruses for use in pathogenesis studies and as new viral vaccine vectors.

Keywords

arenavirus; reverse genetics; Pichinde virus

1. Introduction

Pichinde virus (PICV) is an enveloped RNA virus, belonging to the *Arenaviridae*. The genome of PICV, like those of other known arenaviruses, consists of two single-stranded ambisense RNAs: the large (L) segment of ~7.2 kb and the small (S) segment of ~3.4 kb (1). The L RNA segment encodes the RNA-dependent RNA polymerase L protein in a negative orientation and a small multifunctional Z protein in a positive sense. The S RNA segment encodes another multifunctional protein known as the nucleoprotein (NP) in a negative orientation and the envelope glycoprotein precursor GPC in a positive sense (Fig. 1A). At

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both ends of each of the genome segments, there are 19 nucleotides (nt) that are imperfectly complementary to each other and are predicted to form the panhandle structures that serve as the *cis*-acting elements required for viral RNA transcription and replication. A unique feature of the arenavirus genomic RNA is the noncoding intergenic regions (IGR) located between the two open reading frames. The IGRs range from 59 nt to 217 nt in length and are predicted to form one to three energetically stable stem-loop structures that are proposed to contribute to the termination of transcription.

The first molecular clone of arenavirus was developed for the prototype lymphocytic choriomeningitis virus (LCMV) (2,3). This reverse genetics (RG) system has served as an invaluable tool to study the biological functions of arenavirus proteins and viral RNA elements (e.g., IGR). In this chapter, we describe two different RG systems for PICV. The bi-segmented RG system generates recombinant PICV with two genomic RNA segments (L and S), whereas the tri-segmented RG system produces viruses with three segments (L and S1 and S2) (Fig. 1B). A similar strategy to generate the tri-segmented genome of LCMV has also been recently developed by Emonet and colleagues (4). In our studies, we have shown that recombinant PICV generated from the bi-segmented RG system can recapitulate the parental (stock) viruses in terms of viral growth kinetics in vitro and virulence in vivo (5,6). On the other hand, recombinant PICV generated from the tri-segmented RG systems are highly attenuated and therefore can be used as vaccine vectors to deliver foreign antigens (7).

2 Materials

2.1 Plasmids, cell lines and media

1. Plasmids: pUC19-HDVT7t vector were used for cloning PICV genome L segment and S segment, respectively (4). NP and L gene were cloned into pCAGGS mammalian vector under CMV promoter.
2. Cell lines: BSRT7–5 cells, which constitutively express the T7 RNA polymerase, were obtained from K. Conzelmann at Ludwig-Maximilians-Universität, Germany. BHK21 cells were used for PICV amplification. Vero cells were used for PICV plaque assay.
3. Culture Media: BSRT7–5 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mg geneticin per ml, and 50 mg penicillin and streptomycin per ml. BHK21 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 mg penicillin and streptomycin per ml. Vero cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 50 mg penicillin and streptomycin per ml.
4. Opti-MEM (Invitrogen) for use in transfections and for culturing transfected cells.

2.2 Reagents and buffers

1. Lipofectamine2000 (Invitrogen).

2. Neutral red dye (Sigma).
3. Phosphate Buffer Saline (PBS) (Sigma).
4. Geneticin G418 (Gibco).
5. 2% Agar Solution: Dissolve 2 grams of agar in 100 ml of distilled water. Autoclave it.
6. First agar overlay: 0.6 ml of 2% agar and 2.4 ml of complete MEM.
7. Second agar overlay: 0.4 ml of 2% agar, 1.6 ml of complete MEM and 120 μ l of 0.33% neutral red dye.
8. 0.45 μ m filter (Millipore).
9. Sharpie pen for demarcating plaques on bottom of plastic plates.

2.3 Equipment

1. Biosafety cabinet that is Class II, type A2 (air re-circulates air after HEPA filtration) (ThermoFisher).
2. CO₂ incubator MCO-18AC(UV) (Sanyo).
3. Speed Pipettor (Eppendorf).
4. Water Bath (ISOTEMP 210, Fisher).
5. Microwave oven.

3 Methods

3.1 Generation of the bi-segmented and tri-segmented PICV RG systems

1. Seed 4×10^5 BSRT7–5 cells/well in 6 well plates at 24 h prior to transfection. (*see Note 1*)
2. Replace cell-culture supernatant with a fresh aliquot of the complete DMEM medium (without Geneticin) prior to transfection. (*see Note 2*)
3.
 - a. For bi-segmented PICV system, mix 4 μ g of the PICV L segment plasmid and 2 μ g of the PICV S segment plasmid in 100 μ L Opti-MEM (*see Note 3*).
 - b. For tri-segmented PICV system, mix 2 μ g of the PICV L segment plasmid and 1 μ g of each of the PICV S1 and S2 segment plasmids in 100 μ L Opti-MEM (*see Note 4*).

¹.Use low passaged BSRT7–5 cells for optimal transfection efficiency.

².Maintain the BSRT7–5 cells in a medium free of any antibiotics for at least 24 hrs prior to transfection.

³.Using the bi-segmented PICV RG system, recombinant viruses of two different strains (P2 and P18) were generated. Recombinant P2 viruses (rP2) produced smaller plaque sizes than the rP18 viruses, and grew slower with less viral titers than rP18 viruses in cell cultures (Fig. 2).

⁴.Using the tri-segmented PICV RG system, recombinant viruses that carry 3 genomic segments were generated. Recombinant viruses with wild-type rP18 sequences replicated to a higher levels in cell cultures than rP18tri-GFP

4. Add 9 μL of Lipofectamine2000 in 100 μL Opti-MEM in a separate tube ($\mu\text{g DNA} \times 1.5 = \mu\text{L Lipofectamine2000 needed}$).
5. Incubate the above two mixtures separately for 5 min at ambient temperature.
6. Mix the two mixtures (Opti-MEM/plasmid and Opti-MEM/Lipofectamine2000), vortex the sample well and incubate the sample for 5 min at ambient temperature.
7. Dropwise add 200 μL of the sample onto the cells.
8. At 4 h post-transfection, replace the cell medium with a fresh aliquot of the complete DMEM medium (without Geneticin).
9. Collect 1 ml of the cell-culture supernatant at 48 h and 72 h post-transfection for plaque assay (below) and at each time add an equal fresh aliquot of DMEM medium (without any antibiotics) to the cells.

3.2 Plaque assay and plaque purification of recombinant viruses.

1. Seed 300,000 Vero cells/well in 6 well plates (using the complete MEM medium)
2. Prepare 10x serially diluted virus samples (above) in MEM media (without FBS and antibiotics) (*see Note 5*)
3. Aspirate medium from cells and wash the cells once with PBS.
4. Add 500 μL of the diluted virus samples to the cells and incubate the cells at 37°C for 60 min with intermittent gentle rocking of the plate to spread the virus uniformly.
5. After 60 min of virus adsorption, aspirate the virus from the cells and wash cells with PBS.
6. Slowly add the first agar overlay onto the cells (*see Notes 6, 7 and 8*). (Calculate the volume of overlay needed and mix the medium and 2% agar in appropriate ratios as described in the Materials section).
7. Incubate the plates for 4 days at 37°C with 5% CO_2 .
8. After 4 days of incubation, add the second agar overlay onto the cells and let it solidify for 5–10 min. (*see Note Notes 6, 7 and 8*).
9. Incubate the plates overnight at 37°C and count the plaques the following day.
10. To pick a plaque for further virus amplification (below), choose a plaque that is not touching any others and mark it on the bottom of plate with a Sharpie pen.

⁵. Change the pipet tips at each dilution or use the speed pipettor to prepare the serial dilutions. This helps eliminate any human errors in pipetting.

⁶. Overlay media should be added immediately after the PBS wash in order to make sure that the cells do not get dried out.

⁷. In order to maintain the flowing consistency of the overlay medium for prolonged duration, keep the medium in 37°C water bath prior to mixing it with the 2% molten agar. Keep the molten agar in 65°C water bath.

⁸. Do not immediately cover the plates after adding the overlay media in order to avoid accumulation of water condensations.

11. Puncture the agar using the P1000 pipetman and insert the tip straight down to the plaque to aspirate a small volume of the medium that contains the virus. Dispense the virus sample into 1 mL of MEM, and pipet up and down to remove the agar from the pipet tip (*see* Note 9)

3.3 PICV amplification

1. Seed 8×10^5 BHK-21 cells in a 10 cm dish at 24 h prior to infection.
2. Infect the cells by adding 1 mL of the plaque-purified virus to the cells for 60 min (*see* Note 10)
3. Remove the virus by aspiration and add 10 mL of complete DMEM medium to the cells.
4. After 48 h, collect the cell-culture supernatant, and filter it through a 0.45 μ m filter.
5. Prepare small aliquots of the virus (0.5–1 mL) and store at -80°C . (*see* Note 11).
6. Determine the virus titer by plaque assay as described in section 3.2 above.

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⁹·In order to pick up a purified plaque, cut the mouth of a 1000 μ L tip with a sterile razor. Broaden mouth tip can more easily aspirate a chunk of semi-solidified agar.

¹⁰·Swirl the plate every 10 mins to ensure uniform distribution of virus over the surface of the cell monolayer.

¹¹·Make smaller aliquots in order to avoid repeated freeze-thawing of the virus stocks which may drastically impact the accuracy of the virus titers.

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Figure 1. Schematic diagram of the bi-segmented and tri-segmented PICV reverse genetics systems:

(A) Wild type (WT) bi-segmented rP18 PICV genome comprised of L and S segments (B) tri-segmented rP18tri PICV genome comprised of L, S1 and S2 segments encoding eGFP reporter gene. IGR: intergenic region, NP: nucleoprotein, GPC: glycoprotein complex, eGFP: green fluorescence protein, MCS: multiple cloning site

Figure 2. Comparison of bi-segmented PICV viruses:
(A) Plaque sizes of the rP2 and rP18 wild type viruses (B) Growth kinetics of the rP2 and rP18 bi-segmented Pichinde viruses.

A.

B.

Figure 3. Growth kinetics of bi-segmented and tri-segmented PICV:
 (A) Plaque sizes of rP18 (WT) and rP18tri-GFP PICV (B) Analysis of growth kinetics of the rP18 and rP18tri-GFP in BHK-21 cells at MOI of 0.1