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Development of reverse genetics system for small ruminant morbillivirus: Rescuing recombinant virus to express *Echinococcus granulosus* EG95 antigen

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ABSTRACT

Peste des petits ruminants and cystic hydatidosis may be simultaneously endemic in a given area. Their pathogens are small ruminant morbillivirus (SRMV) and *Echinococcus granulosus* (*E. granulosus*), respectively. The SRMV, formerly called peste-des-petits-ruminants virus (PPRV), is classified into the genus *Morbillivirus* in the family *Paramyxoviridae*. This virus is an ideal vaccine vector to deliver immunogenic proteins. In this study, a reverse genetics system was developed to rescue a recombinant SRMV (Nigeria 75/1 strain) expressing *E. granulosus* EG95 antigen *in vitro*. The recombinant SRMV, albeit replicating more slowly than its parental virus, could effectively express the EG95 antigen in cells by analyses of Western blot, indirect immunofluorescence and mass spectrometry. An EG95 subunit vaccine has been widely used for prevention of cystic hydatidosis in some areas of China. The EG95-expressing SRMV, if proven to induce effective immune responses against both diseases in a future animal experiment, would become a potential candidate of bivalent vaccine.

1. Introduction

Peste des petits ruminants (PPR) is a highly contagious disease, which mainly affects goats and sheep, occasionally wild small ruminants and even large ruminants (Abubakar et al., 2017; Khalafalla et al., 2010; Zakian et al., 2016). This disease is included in the OIE (World Organization for Animal Health) list of notifiable terrestrial animal diseases. Its clinical signs are usually characterized by pyrexia, mucopurulent oculo-nasal discharges, diarrhea, stomatitis and pneumonia (Jagtap et al., 2012; Truong et al., 2014).

The etiological agent of PPR is peste-des-petits-ruminants virus (PPRV), which however has been renamed small ruminant morbillivirus (SRMV), according to the latest virus taxonomy of the International Committee on Taxonomy of Viruses (Liu et al., 2018b). SRMV is classified into the genus *Morbillivirus* in the family *Paramyxoviridae*, and its genome is a single strand of RNA with negative polarity (Mahapatra et al., 2006), which encodes six structural (N, P, M, F, H and L) and two nonstructural (V and C) proteins in the order of 3′-N-P (V/C)-M-F-H-L-5′. Viral RNA genome is encapsidated by the N protein forming a helical nucleocapsid, in combination with the RNA-dependent RNA polymerase (L protein) and the P protein to form a ribonucleoprotein complex (Parida et al., 2015).

The first PPR vaccine strain (Nigeria 75/1) was developed by serial passages of SRMV in cell culture. The Nigeria 75/1 strain has been mostly used with high efficacy in sheep and goats, conferring immune protection at 1 month post vaccination with a single dose (Khan et al., 2009). This protection can usually remain for at least 1 (Rashid et al., 2010) and probably 3 years without side reactions normally noticed. The Nigeria 75/1 strain, if genetically modified by reverse genetics tool to express a foreign antigen, would play a potential role in development of bivalent vaccines against PPR and other diseases (Yin et al., 2014). Although many attempts had been made to underpin its development (Bailey et al., 2007; Minet et al., 2009; Zhai et al., 2010), a system of SRMV reverse genetics was unavailable until reported recently by two groups (Hu et al., 2012; Muniraju et al., 2015), both of which independently rescued recombinant SRMVs from individual cDNA clones but used different promoters (CMV and T7 promoters) to drive transcription of a full-length virus antigenome.

Cystic hydatidosis is a zoonotic disease caused by the taeniid cestode parasite *Echinococcus granulosus* (*E. granulosus*). Goats and sheep act as intermediate hosts for the parasite, while canids are the definitive hosts (Jabbar et al., 2011). EG95, a recombinant antigen from the parasite egg (oncosphere), can confer a considerably high level of protection (96–100%) in vaccinated animals (Heath et al., 2003; Lightowlers et al., 1999, 1996). An EG95 antigen-based subunit vaccine has been commercially available in China, and can induce a robust immune response in goats and sheep. In this study, a reverse genetics system was successfully developed for recovery of recombinant SRMV

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Nigeria 75/1 strain. A recombinant SRMV was rescued by this system and proven to have an ability to express the EG95 antigen *in vitro*.

2. Materials and methods

2.1. Cells and viruses

The BSR-T7/5 cells and Vero-Dog-SLAM (VDS) cells were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, USA), and containing penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (0.25 μg/mL) and G418 (500 μg/mL). A recombinant SRMV Nigeria 75/1 strain (rSRMV) without any modification in its genome was rescued previously using reverse genetics at our laboratory.

2.2. Construction of plasmids

A genome sequence of Nigeria 75/1 strain (Genbank access No.: KY628761) was used for designing a full-length rSRMV cDNA clone containing an open reading frame (ORF) of EG95 antigen (Genbank access No.: AY421719). This ORF was flanked by the Kozak sequence (Kozak, 1987) at its 5′ end to improve protein expression possibly, and meanwhile, the "ACT" encoding the last amino acid at its 3′ end was deleted to obey the "rule of six" of paramyxovirus (Kolakofsky et al., 2005). In order to facilitate insertion of another sequence into the cDNA clone, the EG95 ORF was flanked by *Not* I and *Pme* I sites. The modified EG95 ORF was regulated both by an M gene start (GS) sequence and by a P gene end (GE) sequence. In the upstream region of cDNA clone, an autocatalytic hammerhead ribozyme (Hh-Rbz) sequence was introduced between the T7 promoter and the cDNA clone. Additionally, three guanine (G) residues were added to the 3′ end of T7 promoter sequence to enhance transcription efficiency (Martin et al., 1988). In the downstream region of cDNA clone, a hepatitis delta virus ribozyme (HDV-Rbz) sequence was introduced between the cDNA clone and the T7 terminator sequence. The 5′-and 3′-end-modifying rSRMV-EG95 cDNA clone (Fig. 1) was chemically synthesized and subcloned into the pBR322 plasmid.

Three ORFs coding for N, P and L proteins were subcloned into the mammalian expression vector (pCAGGS), respectively. Three recombinant plasmids, pCAGGS-N, pCAGGS-P and pCAGGS-L (Fig. 1), served as helper plasmids for rescue of recombinant SRMV expressing EG95 antigen (rSRMV-EG95). The reverse genetics system of SRMV contained four recombinant plasmids: rSRMV-EG95 cDNA clone, pCAGGS-N, pCAGGS-P and pCAGGS-L, all of which were purified using a PureLink™ HiPure Plasmid Maxiprep Kit (Thermo Fischer, Carlsbad, USA) according to the manufacturer's instruction.

2.3. Rescue of recombinant virus

BSR-T7/5 cells (Buchholz et al., 1999) were seeded into a six-well plate 1 d before co-transfection. Twenty-four hours later, cell monolayers at 70–90% confluency were co-transfected with the rSRMV-EG95 cDNA clone (2.5 μg/well), pCAGGS-N (1.5 μg/well), pCAGGS-P (1.0 μg/well) and pCAGGS-L (1.0 μg/well) using Lipofectamine 2000 (Thermo Fischer, Carlsbad, USA) according to the manufacturer's instruction. Transfected cells were cultured at 37 °C with 5% CO_2 in DMEM supplemented with 10% FBS but without G418. BSR-T7/5 cell cultures were harvested 5–7 d post co-transfection, and subjected to one freeze-and-thaw cycle for collecting supernatant to infect VDS cells for 3–5 h. The supernatant was subsequently replaced with growth medium with G418 for further incubation at 37 °C. The rescued rSRMV-EG95 was subjected to serial blind passages in VDS cells. When a typical cytopathic effect (CPE) (*e.g.*, syncytium formation) appeared during blind passage, the rSRMV-EG95 would be passaged for further generations.

2.4. RT-PCR analysis of rSRMV-EG95

The culture supernatant of rSRMV-EG95 at passage 5 was harvested for extraction of viral RNA by a High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany). The extracted RNA was used as template for RT-PCR analysis using the PrimeScript™ One Step RT-PCR Kit (Takara, Dalian, China). The forward primer (P-f: 5′-AGCCATTCTTGCCAAGCAGCCGTAA-3′) targeted the downstream region of P ORF, and the reverse primer (P-r: 5′-TATCAAAATCGTAGATCTCGGTCAT-3′) targeted the upstream region of M ORF (Fig. 1). The RT-PCR reaction underwent 50 °C for 30 min, 94 °C for 2 min and then 30 cycles at 94 °C (30 s), 55 °C (30 s) and 72 °C (50 s) in a thermocycler. To eliminate a false-positive result caused by residual contamination of the rSRMV-EG95 cDNA clone, the extracted RNA was simultaneously subjected to PCR analysis as a control using the same primer pairs. The PCR reaction contained 2 × PrimeSTAR Max Premix (Takara, Dalian, China) and underwent 35 cycles at 98 °C (10 s), 55 °C (10 s) and 72 °C (10 s). RT-PCR and PCR products were detected by agarose gel electrophoresis, followed by Sanger sequencing to confirm it.

2.5. Growth curve of rSRMV-EG95

Growth kinetics of the passage-7 rSRMV-EG95 was compared with that of the passage-7 rSRMV *in vitro*. Briefly, VDS cells were plated into two T-25 flasks (4×10^6 cells/flask) for incubation at 37 °C up to 2 h. Both rescued viruses were inoculated into individual flasks (MOI = 0.01) for incubation at 37 °C. At 0, 3, 24, 48, 72 and 96 h post infection, 100 μL supernatants from infected cells were collected immediately for viral titration by 50% tissue culture infective dose ($TCID_{50}$) assay, respectively. The viral titer for each sample was calculated by the Spearman-Kärber equation (Finney, 1952).

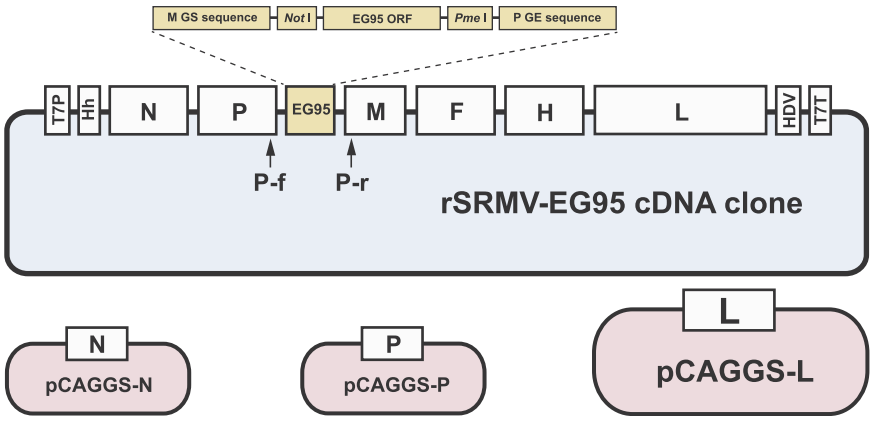

Fig. 1. Schematic representations of the rSRMV-EG95 cDNA clone, pCAGGS-N, pCAGGS-P and pCAGGS-L. The 5′- and 3′-end-modifying rSRMV cDNA clone was chemically synthesized and subcloned into the pBR322 plasmid. Three ORFs coding for N, P and L proteins were subcloned into the pCAGGS plasmid, respectively. One pair of primers, P-f (5′-AGCCATTCTTGCCAAGCAGCCGTAA-3′) and P-r (5′-TATCAAAATCGTAGATCTCGGTCAT-3′), was designed for RT-PCR analysis on rescue of rSRMV-EG95. GS: gene start; GE: gene end; ORF: open reading frame; T7P: T7 promoter with three extra G; Hh: hammerhead ribozyme; HDV: hepatitis delta virus ribozyme; T7T: T7 terminator.

2.6. Western blot analysis of EG95 antigen

Expression of the EG95 antigen was assessed by Western blot. Briefly, VDS cells cultured in flasks were infected either with rSRMV-EG95 or with rSRMV, and were harvested at 72 h post infection. Subsequently, cells were mixed with gel loading buffer for incubation at 95 °C for 10 min. The denatured samples were analyzed by SDS–PAGE in a 12% acrylamide gel, and then transferred to a nitrocellulose membrane. Blots were incubated with blocking solution (Sangon Biotech, Shanghai, China) for 1 h, followed by incubation with the anti-EG95 polyclonal antibodies (pAb) (Liu et al., 2018a) for 2 h. The membrane was washed three times with TBST for 30 min, and then incubated with anti-mouse IgG-peroxidase (Sigma, St. Louis, USA) for 1 h. The membrane was washed as above and subjected to incubation with DAB solution (Thermo Fischer, Carlsbad, USA) for 3 min. The reaction was stopped with ultra pure water.

2.7. Indirect immunofluorescence assay of EG95 antigen

Expression of the EG95 antigen was confirmed by indirect immunofluorescence assay (IFA). Briefly, VDS cells were infected with rSRMV-EG95 and rSRMV for 36 h, respectively, and then fixed in 4% paraformaldehyde at room temperature for 30 min. After fixation, cells were washed four times with PBS, and then permeated with 0.4% Triton X-100 at room temperature for 30 min. After permeation, cells were washed three times with PBS and blocked in blocking solution (Sangon Biotech, Shanghai, China) at 37 °C for 1 h. Subsequently, cells were incubated with the anti-EG95 pAb (1: 400 in blocking solution) at 37 °C for 1 h. After incubation with the primary antibody, cells were washed three times with PBS and incubated with two different fluorescein-conjugated antibodies (Alexa Fluor® 488 conjugate and Alexa Fluor® 555 conjugate, Thermo Fischer, Carlsbad, USA) (1: 250 in blocking solution) at 37 °C for 1 h, respectively. Cells were washed three times with PBS, coated with 90% glycerin, and visualized under the fluorescence microscope.

2.8. Mass spectrometry of EG95 antigen

Expression of the EG95 antigen was confirmed further by mass spectrometry. VDS cells were harvested at 72 h post infection with rSRMV-EG95 for SDS–PAGE analysis in a 12% acrylamide gel. An expected EG95 band was cut out for mass spectrometry at the Beijing Protein Innovation Co., Ltd (Beijing, China).

3. Results

3.1. Rescue of recombinant virus

To rescue viable recombinant virus, the transfection experiment was carried out using the T7 RNA polymerase (T7 Pol)-expressing BSR-T7/5 cells, on which the SRMV infection-like CPE however was unobservable, due to the absence of SRMV receptors surrounding cellular envelope. Therefore, the SRMV infection-permissive VDS cells were used for blind passages of rescued viruses. The specific CPE was invisible until 48 h post the third passage: a typical syncytium formation appeared on the VDS cell monolayer (Fig. 2A, upper left). Such a cell-to-cell fusion was exacerbated over time, and the typical syncytium was replaced with a hyperfusogenic phenotype at 120 h post the third passage on VDS cells (Fig. 2A, upper right). As a mock control, the non-SRMV-infected VDS cells never showed any similar phenotypes at all (Fig. 2A, lower left and right).

3.2. RT-PCR analysis

Total RNA was extracted from rSRMV-EG95 at the fifth passage and analyzed by RT-PCR to confirm its identity. An expected band of amplicon size (739 bp) was observed only on the RT-PCR lane by agarose gel electrophoresis (Fig. 2B, Lane RT-PCR). As a control, PCR analysis (Fig. 2B, Lane PCR) showed no cDNA clone contamination affecting RT-PCR detection. In addition, the Sanger sequencing showed that the passage-5-based RT-PCR product was identical to the 739-bp sequence.

3.3. Growth curve

At the indicated time points, the supernatants from infected cells were collected and titrated in VDS cells. The growth curve of passage-7 rSRMV-EG95 was compared with that of the passage-7 rSRMV (Fig. 2C). Both viruses, albeit identified as nearly identical titers at 0 and 3 h, showed different growth kinetics in the next few days. The rSRMV-EG95 replicated more slowly than the rSRMV after 24 h post infection. Especially at 48 and 96 h post infection, the titer ($TCID_{50}$/mL) difference between both viruses was about ten times.

3.4. Western blot analysis

Expression of the EG95 antigen by the passage-5 rSRMV-EG95 was assessed by Western blot analysis, showing expected bands (Fig. 2D, Lane rSRMV-EG95) of molecular weight (16 kD) on a nitrocellulose membrane. In contrast, rSRMV infection as a control did not result in any EG95 blot-like bands on the membrane at all (Fig. 2D, Lane rSRMV), therefore excluding the possibility of non-specific binding of anti-EG95 pAb to other proteins.

3.5. Indirect immunofluorescence assay

In order to confirm expression of the EG95 antigen, the IFA was performed using the anti-EG95 pAb and two different fluorescein-conjugated antibodies. Bright green (Fig. 3a and c) and red (Fig. 3e and g) syncytia were visible on the Alexa Fluor® 488- and 555-treated cell monolayers, respectively. As a control, rSRMV-infected cells showed neither green (Fig. 3i) nor red (Fig. 3k) phenotype. Therefore, the IFA result confirmed expression of the EG95 antigen *in vitro*.

3.6. Mass spectrometry

Expression of the EG95 antigen was confirmed further by mass spectrometry. Four selected peptide mass maps were illustrated in Fig. 4B (Calculated pI value: 9.30), and totally matched with their corresponding amino acid sequences in the EG95 antigen (Fig. 4A), further confirming expression of the EG95 antigen.

4. Discussion

Although effective systems of reverse genetics were established long ago for other morbilliviruses (Baron and Barrett, 1997; Gassen et al., 2000; Radecke et al., 1995), a platform to rescue SRMV was unavailable until reported recently by two independent groups (Hu et al., 2012; Muniraju et al., 2015). How to construct a SRMV cDNA clone is one of the greatest difficulties in establishing a system of SRMV reverse genetics. Undesirable sequence mutations and (or) deletions sometimes occur during plasmid replication in *E. coli*. The L gene sequence is occasionally unstable during subcloning, and its partial sequences can be irregularly deleted for unexplained reasons. In additional, single-nucleotide mutations would tend to appear in GC-rich regions between the M and F ORFs. These regions may be a potential bottleneck for constructing a SRMV cDNA clone.

To overcome above-mentioned difficulties, a low-copy-number plasmid (pBR322) was used in this study. Alternatively, some *E. coli* competent cells, if characterized by reducing the copy number of plasmids in them, would be recommended for use in cDNA clone construction. However, both strategies are not recommended to be

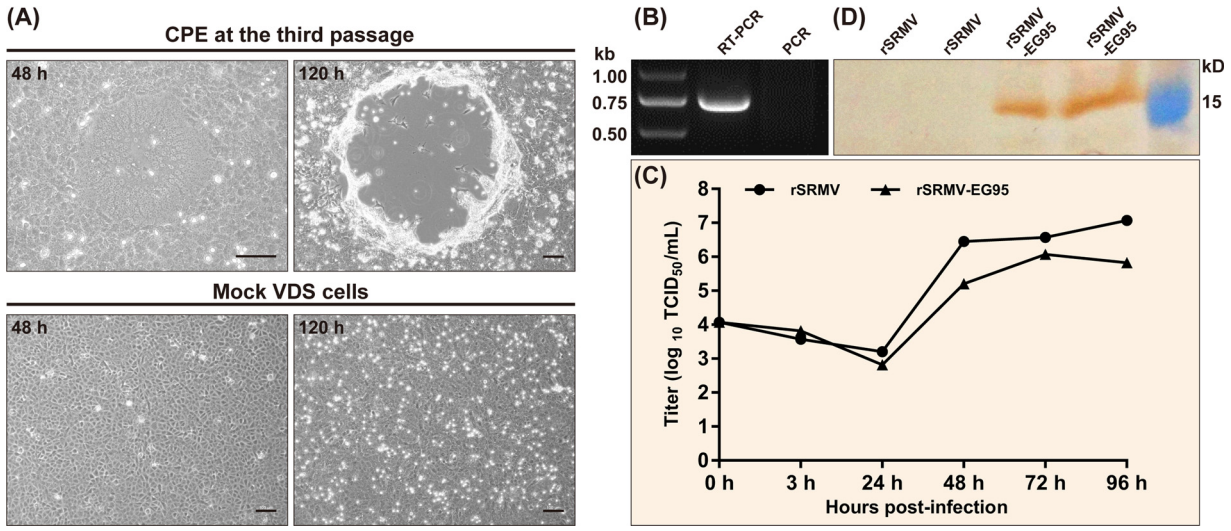

Fig. 2. Passage and characterization of rSRMV-EG95. The rSRMV-EG95 was subjected to serial blind passages in VDS cells. A typical syncytium formation (A, upper left) appeared at 48 h post the third passage, and was replaced with a hyperfusogenic phenotype (A, upper right) on the fifth day. The non-SRMV-infected VDS cells never showed any similar phenotypes at all (A, lower left and right). Bar = 100 μm. Total RNA was extracted from the rSRMV-EG95 at the fifth passage for RT-PCR analysis. An expected 739-bp band of amplicon size was observed only on the lane RT-PCR (B), and as a control, PCR analysis (B) showed no cDNA clone contamination affecting RT-PCR detection. The growth curve of passage-7 rSRMV-EG95 was compared with that of the passage-7 rSRMV (C). Expression of the EG95 antigen was assessed by Western blot analysis, showing expected 16-kD band on the lane rSRMV-EG95 but not on the lane rSRMV (D).

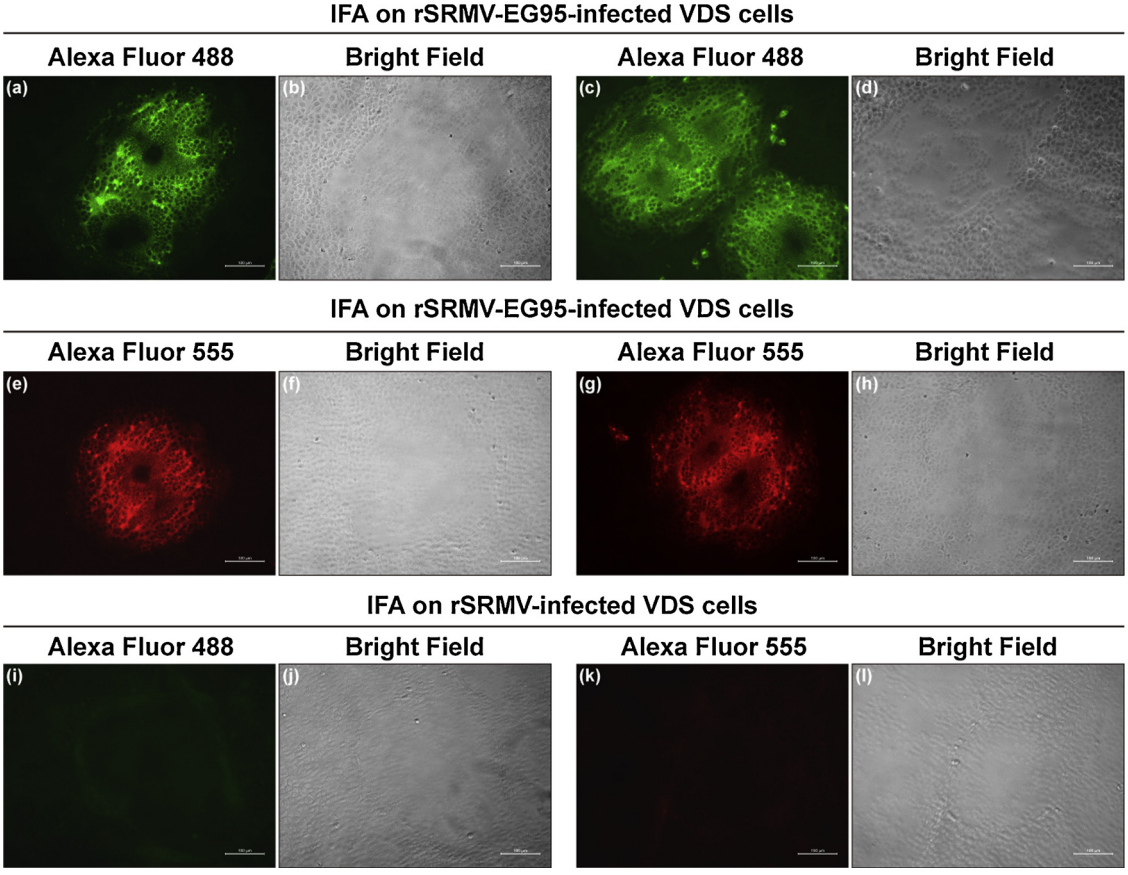

Fig. 3. Indirect immunofluorescence assay on expression of EG95 antigen in VDS cells. Two different fluorescein-conjugated secondary antibodies were used for the indirect immunofluorescence assay, respectively. Bright green (a and c) and red (e and g) syncytia were visible on the Alexa Fluor® 488- and 555-treated cell monolayers, respectively. As a control, rSRMV-infected cells showed neither green (i) nor red (k) phenotype. Bar = 100 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

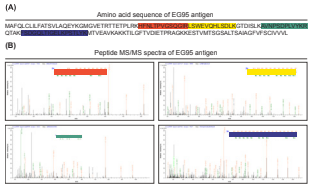

Fig. 4. Amino acid sequence of EG95 antigen and four selected peptide MS/MS spectra. The last amino acid (threonine) was deleted from the full-length EG95 antigen to obey the "rule of six" of paramyxovirus for virus rescue (A). Four selected peptides in the MS/MS spectra, as illustrated by four colorful shades (B), totally matched with their corresponding amino acid sequences (A) in the EG95 antigen. Calculated pI value: 9.30.

simultaneously used, since the copy number may be too low to extract high-quality plasmids from *E. coli*. Another feasible way to reduce the mutation rate is low-temperature (30 to 32 °C) culture of *E. coli* for cDNA clone replication. Even though above-recommended methods are used, sequence mutations in a cDNA clone are still unpredictable in *E. coli*, and therefore its full-length sequencing should be performed before rescuing viruses.

To enhance transcription efficiency of the T7 Pol, the "GGG" were added directly to downstream of the T7 promoter sequence. To generate two correct termini of genome for virus rescue, the recombinant cDNA clone was flanked by the Hh-Rbz and HDV-Rbz sequences (Beaty et al., 2017). However, it had been proven that the Hh-Rbz was non-essential for rescuing recombinant SRMV at our laboratory: a green fluorescent protein (GFP)-expressing SRMV could also been efficiently rescued in absence of the Hh-Rbz. The size of terminator sequence for the T7 Pol is about 139 nt, which may vary depending on the system (Molouki and Peeters, 2017).

As to insertion sites for a foreign gene, there are theoretically seven sites that can be used in a parental SRMV cDNA clone. However, it is difficult to determine which one is optimal both for recombinant virus growth and for foreign protein expression. Especially for the expression of foreign antigen, a high expression level is not always advantageous in immune response induction *in vivo*. In other words, a foreign antigen should be expressed at a proper, but not high, level. To date, the P/M intergenic region in paramyxoviruses has been widely used for insertion of foreign antigens into cDNA clones (Banyard et al., 2010; Cantarella et al., 2009; Marsh et al., 2013; Wang et al., 2012; Xu et al., 2017), and so has this region in this study (Fig. 1).

It is usually considered that designing and constructing paramyxovirus cDNA clones are two most significant determinants for recovery of recombinant viruses. How to construct three helper plasmids is an often neglected but important issue. On the one hand, some commercialized expression plasmids, because containing the T7 promoter sequence, would be regulated by the T7 Pol to initiate an unwanted transcription in BSR-T7/5 cells, possibly reducing the rescue efficiency. Therefore, the T7 promoter sequence-free plasmid, pCAGGS, was used to construct three help plasmids in this study. On the other hand, non-homologous helper plasmids with the rSRMV cDNA clone would also reduce the rescue efficiency. We found that when lineage IV-origin helper plasmids were used for lineage II SRMV recovery, the rescue efficiency was unsatisfactory, compared with using homologous counterparts. Such a low efficiency may be attributed to that homologous helper proteins are more efficient mediators for transcription and replication than the heterologous proteins (Bailey et al., 2007).

The T7 promoter is most frequently used in development of paramyxoviral reverse genetics, because identified by the T7 Pol to initiate non-nuclear transcription, similar to the scenario of paramyxoviral transcription in cytoplasm. In contrast, the CMV promoter is identified by RNA polymerase II (Pol II) to initiate nuclear transcription. A CMV promoter/Pol II-based reverse genetics system may theoretically cause a lower rescuing efficiency than a T7 promoter/T7 Pol-based system, because the former system-driven viral antigenome must be transported into cytoplasm *via* a nuclear pore for viral assembly, but the latter system-driven product can directly be modified in cytoplasm. Simply put, the Pol II system may be more suitable than the T7 Pol system for rescuing RNA viruses with a nuclear replication strategy (Martin et al., 2006; Yanai et al., 2006). However, it has been reported that the Pol II system can also be used to rescue non-nuclear replicating RNA viruses (Hu et al., 2012; Inoue et al., 2003; Li et al., 2011). Even so, the T7 Pol system was still used in this study.

Rescue of paramyxoviruses generally relies on the T7 Pol supplied either by stably transfected helper cells (Buchholz et al., 1999; Mebatsion et al., 2001) or by recombinant helper viruses (Baron and Barrett, 1997; Peeters et al., 1999). Although the T7 Pol-expressing BSR-T7/5 cell line was used to rescue the rSRMV-EG95, SRMV infection-like CPE was never observable on cell monolayer, due to the absence of SRMV receptors. The BSR-T7/5 cells played a role only in rescuing but not in passaging. As mentioned in Subheading 3.1, the syncytium formation was invisible until 48 h post the third passage on VDS cells, whereas the EG95 antigen expression was possibly initiated as early as many days post co-transfection in BSR-T7/5 cells, because we observed several green BSR-T7/5 cells on day 3 post co-transfection in another experiment to rescue GFP-expressing rSRMV. Therefore, serial blind passages in VDS cells are necessary to observe SRMV infection-like CPE. If the syncytium formation is always invisible on VDS cells *via* 5 serial blind passages, recombinant SRMV would not be considered to be rescued from a cDNA clone.

In this study, although SRMV infection-like CPE was always visible after multiple passages in VDS cells, RT-PCR analysis on viral genome was still necessary for confirming recovery of the rSRMV-EG95. To eliminate a possible interference caused by residual rSRMV-EG95 cDNA clone, PCR analysis as a control must be simultaneously performed using the same primer pairs, because sometimes "stubborn" contamination of cDNA clone can be still retained even after several viral passages.

When successfully rescued from a cDNA clone, recombinant morbillivirus generally shows a weak adaptability in cells, owing to a relatively single quasispecies. Its adaptability would be gradually improved with serial passages in cells. Or rather, each viral generation is better than the last in growth kinetics especially during the first passages (Liu et al., 2016). Therefore, the same number of viral passages is an important aspect that should be considered for growth kinetics comparison between rescued viruses. In this study, the passage-7 was chosen for comparing growth curves between the rSRMV-EG95 and the rSRMV. In the rSRMV-EG95 genome, the EG95 sequence plays a role of nonstructural gene in viral replication. Its introduction, more or less, can affect the efficiency of viral growth. The EG95 sequence is located between the P and M genes (Fig. 1), and therefore would affect transcription of the M, F, H and L genes at some extent, due to the characteristics of mRNA transcription gradient of morbilliviruses (Rima and Duprex, 2006). Indeed, the rSRMV-EG95 grew more slowly than the rSRMV after a lag period (Fig. 2C). It will be one of challenges to increase the final titer of rSRMV-EG95 on the road to vaccine commercialization.

Expression of the EG95 antigen *in vitro* was assessed by Western blot, and confirmed by IFA and mass spectrometry, demonstrating that the reverse genetics system was efficient to rescue recombinant SRMV expressing a foreign protein. Before this study, the EG95 antigen had also been expressed by recombinant vaccinia virus (Dutton et al., 2012), orf virus (Marsland et al., 2003; Tan et al., 2012) and goatpox virus (Liu et al., 2018a). Moreover, its expression by viral vectors had been proven to induce effective immune responses *in vivo* (Dutton et al., 2012; Tan et al., 2012). The live-attenuated SRMV vaccine and the EG95 subunit vaccine are generally used for prevention of PPR and cystic hydatidosis, respectively. A bivalent vaccine against both diseases has not been commercially available in China as yet. The recombinant SRMV has been proven to be a powerful vaccine vector to deliver foreign antigen *in vivo* (Yin et al., 2014). If proven to be efficient

in resisting both diseases in future animal experiments, the rSRMV-EG95 would become a potential candidate of bivalent vaccine.

Acknowledgments

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