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Original article

Optimal expression and purification of sapelovirus A structural protein VP1, and its immunogenicity in mice

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Abstract

Sapelovirus A (SV-A) is a positive-sense single-stranded RNA virus which is associated with acute diarrhea, pneumonia and reproductive disorders. The virus capsid is composed of four proteins, and the functions of the structural proteins are unclear. In this study, we expressed SV-A structural protein VP1 and studied its antigenicity and immunogenicity. SDS-PAGE analysis revealed that the target gene was expressed at high levels at 0.6 mM concentration of IPTG for 24 h. The mouse polyclonal antibody against SV-A VP1 protein was produced and reached a high antiserum titer (1: 2,048,000). Immunized mice sera with the recombinant SV-A VP1 protein showed specific recognition of purified VP1 protein by western blot assay and could recognize native SV-A VP1 protein in PK-15 cells infected with SV-A by indirect immunofluorescence assay. The successfully purified recombinant protein was able to preserve its antigenic determinants and the generated mouse anti-SV-A VP1 antibodies could recognize native SV-A, which may have the potential to be used to detect SV-A infection in pigs.

Key words: sapelovirus A, prokaryotic expression, purification

Introduction

Sapelovirus (SV), a new genus of picornaviruses, is a positive-sense single-stranded RNA virus. This genus consists of three species: sapelovirus A (SV-A, formerly named porcine sapelovirus), sapelovirus B (SV-B, formerly known as simian SV) and avian sapelovirus (formerly duck picornavirus TW90A) (Adams et al. 2015). The prevalence of SV-A in infected pigs has been reported in many countries, including Spain (Cano-Gómez et al. 2013), the United Kingdom (Schock et al. 2014), Brazil (Donin et al. 2014) and Korea (Son et al. 2014b). SV-A can replicate in the gastrointestinal epithelia, and infections by SV-A are either asymptomatic or associated with acute diarrhea, polioencephalomyelitis, pneumonia and reproductive disorders (Lan

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et al. 2011). Clinically, co-infection occurs frequently between SV-A and other pathogens, such as teschovirus, enterovirus, coronavirus, astrovirus, circovirus, sapovirus and parvovirus (Shan et al. 2011, Cheung et al. 2013).

The length of the SV-A genome is approximately 7.5-8.3 kb, and contains a single open reading frame (ORF) flanked by non-translated regions at both ends. The ORF consists of four structural proteins (VP4-VP2-VP3-VP1), followed by seven non-structural proteins (2A-2B-2C-3A-3B-3C-3D) (Krumbholz et al. 2002). The VP1, VP2 and VP3 proteins locate at the surface of the virion and exhibit high sequence variability (Sozzi et al. 2010). Additionally, a leader (L) protein locates at the N-terminus of the polyprotein (Hales et al. 2008). At present, studies of SV-A are focused on genome sequence and analysis (Chen et al. 2012, Son et al. 2014a) as well as molecular survey (Prodělalová 2012, Donin et al. 2015). However, the expression and antigenicity of the VP1 protein from SV-A have not yet been reported.

The SV-A structural gene VP1 was therefore expressed in the current study. To optimize the expression levels of recombinant VP1 proteins, different induced concentrations of isopropyl-b-D-thiogalactopyronoside (IPTG) were used and different induced times were allocated. Following expression and purification, a polyclonal antibody against SV-A VP1 protein was produced, and the antigenicity and immunogenicity of the recombinant VP1 protein were detected.

Materials and Methods

PCR amplification and construction of expression plasmid

The SV-A csh strain was isolated in 2011 from PCR-positive fecal samples of pigs on a pig farm and preserved by our laboratory (Lan et al. 2011). Viral RNA was extracted from the SV-A csh strain and used for cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen). Based on the conserved sequences of the SV-A gene (GenBank: HQ875059), a primer of SV-A structural VP1 was designed using Oligo 6.0 software (Molecular Biology Insights, Inc., Cascade, CO). The sense primer was: 5'-CCGCTC GAGGGAGATATAAAGGACATAGTACAAG-3' (Xho I restriction sites), and the antisense primer was 5'-CCGGAATTCCTAAGCTTGGGTTGCAGGGTA-3' (EcoR I restriction sites). PCR reaction mixtures (50 μL) containing 2 μL of cDNA, 2 μL of each primer, 25 μL of PCR premix and 19 μL of ddH_2O were produced as follows: denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 55 s, and a final extension of 72°C for 10 min. The PCR product was purified using Gel Extraction Kit, digested with Xho I and EcoR I, and inserted into the Xho I and EcoR I sites of the pCold I vector using T4 DNA ligase at 37°C for 0.5 h. The recombinant plasmid pCold-VP1 which contained a gene of the correct size was identified by PCR, restriction enzyme digestion and DNA sequence analysis.

Inducible expression and optimization of expression conditions

The recombinant plasmid was transformed into Rosetta competent cells. Then, the single recombinant colony was grown in 5 ml LB medium in the presence of ampicillin (100 μg/ml) at 37°C and 180 rpm. The optical density (OD600) of cell concentration was detected every 15-30 min until the culture reached mid-log growth (OD600 = 0.6 - 0.8), and IPTG was then added to induce protein expression at 15°C for 24 h. To determine the existing form of the protein, the induced products were centrifuged at 4,000 rpm for 10 min and the supernatants were discarded. The precipitate was resuspended using 300 μL PBS, and disintegrated by sonication. The bacterium suspension (50 μL) was then collected, and another suspension was centrifugated (12, 000 rpm, 10 min). The clear supernatant and cell pellet were then collected, respectively. The cell pellet was re-suspended in 100 μL PBS. After adding 25 μL 5 × SDS-PAGE sample buffer and boiling for 10 min, samples were analyzed by SDS-PAGE. Negative control cultures containing the empty pCold vector were processed in parallel. To increase the expression level, inducible conditions were optimized, including the concentrations of IPTG (0 mM, 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM or 1mM) and samples collected at time-points (0 h, 8 h, 16 h, 24 h, 32 h and 40 h) after the addition of IPTG. The pCold vector was also transformed into competent cells and served as control.

Purification of recombinant protein

SV-A VP1 protein was overexpressed in *E. coli* Rosetta by growing in 250 ml of medium at 15°C. Cells were then harvested through centrifugation at 4000 rpm for 10 min. The supernatants were discarded and the pellets were resuspended using 7.5 ml PBS (PH = 7.2), followed by lysing using ultra sonication. The lysates were then centrifuged at 12000 rpm for 10 min at 4°C, and the precipitates were resuspended using 7.5 ml binding buffer containing 100 mM NaH_2PO_4, 10 mM Tris·Cl and 8 M urea. The resulting cell supernatant was injected into an affinity column with 1 ml Iso Ni-NTA Resin at a flow rate of 1 ml/min. The packed column was first washed with a 10-fold volume of binding buffer, and then a 2-fold volume of

wash buffer (100 mM NaH_2PO_4, 10 mM Tris·Cl, 10mM Imidazole and 8M urea). Finally, the bound proteins were eluted with elution buffer (100 mM NaH_2PO_4, 10 mM Tris·Cl, 250 mM Imidazole and 8M urea). Each fraction contained 1 ml of effluent. The assumed peak containing SV-A protein was identified and the effluent was collected. The purified samples were analyzed by 12% SDS-PAGE.

Production of polyclonal antibodies against the recombinant protein

Three male 4-week-old BALB/c mice (16-18 g) were used to produce antibodies against the purified recombinant SV-A VP1 proteins. Pre-immunized mouse serum was used as a negative control. For primary immunization, 75 μg protein per mouse in Freund's complete adjuvant was inoculated subcutaneously on the dorsal back of the mouse. The mouse then received two boosters of 100 μg protein in incomplete Freund's adjuvant at two-week and one-week intervals, respectively. Seven days after the third immunization, the eyeballs of the mice were extracted and about 1 mL blood was collected. The blood was first kept at 37°C for 1 h and then overnight at 4°C to allow clotting. Antiserum was collected by centrifugation at 4,000 rpm for 5 minutes. The protocols for the maintenance and care of experimental animals were approved by the Animal Ethical Committee of Shanghai Jiao Tong University.

Antiserum titer determination by enzyme linked immunosorbent assay (ELISA)

Antibody titer was measured using indirect ELISA as previously described (Wu et al. 2016). Briefly, ELISA plates were coated with purified recombinant protein (40 μg/ml, 100 μl/well) at 4°C overnight. The plates were then washed with PBST (0.05% Tween 20 in PBS) and blocked in PBS containing 5% skimmed milk at 37°C for 1 h. After washing, different dilutions (1:400 to 1: 204,800) of 100 μl polyclonal antibody against SV-A VP1 protein were added to the plates and incubated at 37°C for 1 h. Pre-immunized mouse serum served as negative control. The plates were then washed with PBST and incubated with 100 μl horseradish peroxidase-conjugated (HRP) goat anti-rabbit IgG (1:0000, Sigma). After washing, the o-phenylenediamine substrate solution (100 μl/well) was added and incubated at 37°C for 10 min in the dark. Coloration was stopped with 50 μl H_2SO_4 (2 M) and the absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Model 680, USA). A positive OD > 0.1 and the value of OD positive / OD negative > 2.1 were considered as positive.

Western blot analysis

After the purified SV-A VP1 protein was separated by 12% SDS-PAGE gels, they were transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% skimmed milk in 10 mM Tris-HC1 (pH 8.0), 150 mM NaCl, and 0.05% Tween-20 (TBST) at 37°C for 1 h, and washed three times, followed by incubation with mouse monoclonal anti-His antibodies (1:2000) or SV-A positive serum (1:1000) as the primary antibody at 4°C overnight. After three times washing again, the membrane was incubated with HRP-labeled goat anti-mouse IgG (1: 5,000) as the second antibody at 37°C for 1 h. The membrane was then washed again, and visualized using a Biochemical Imaging System (UVP; Upland, CA, USA).

Indirect immunofluorescence assay (IFA)

To test the specificity of SV-A virus when infecting PK-15 cells, IFA was performed with the SV-A VP1 antiserum. PK-15 cells were seeded into 12-well plates for 24 h until they reached 100% confluence. The cells were then infected with SV-A virus (MOI = 1) for 30 h. After washing three times with PBS, the cells were fixed with 4% paraformaldehyde at room temperature for 10 min, permeabilized with 0.2% Triton X-100, blocked with 5% BSA diluted in PBS, and stained with mouse negative serum or mouse anti-SV-A VP1 polyclonal antibody (1:500) at 4°C overnight. After washing three times with PBS, the cells were incubated with Alexa Fluor® 488 Goat Anti-Mouse IgG (1:200, Yeasen, Shanghai, China) for 45 min at 37°C. The cells were then washed with PBS, incubated with 4,6-diamino-2-phenylindole for 3 min at room temperature and analyzed by fluorescence microscopy.

Results

Construction of recombinant plasmids

The SV-A structural gene VP1 was amplified using specific primers by PCR. After the gene fragments were inserted into the pCold I vector, the recombinant plasmids were confirmed by PCR and digestion. PCR and restriction digestion analysis showed that the expected sizes of VP1 (855 bp) were found (Fig. 1), suggesting that recombinant gene expression plasmids were successfully constructed.

Inducible expression and optimal conditions for protein expression

The recombinant gene plasmid or pCold I plasmid was transformed into Rosetta strain, induced with

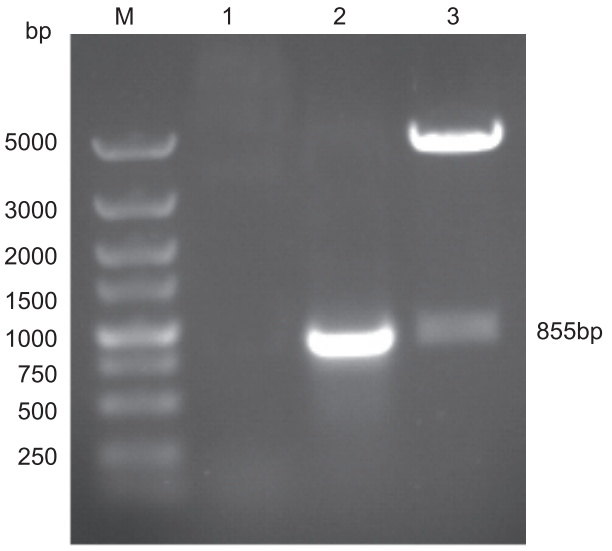

Fig. 1. Confirmation of SV-A VP1 gene recombinant plasmid by PCR and restriction digestion. M, DNA marker 5000. Lane 1: PCR products of negative control; Lane 2: PCR products of recombinant plasmid; Lane 3: Recombinant plasmid was digested with Xho I and EcoR I.

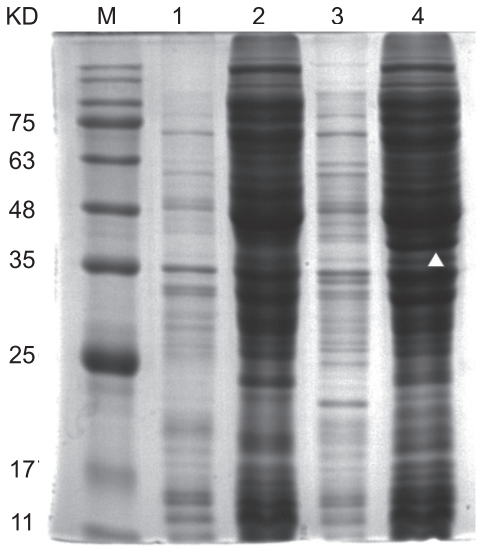

Fig. 2. SDS-PAGE analysis of expression of recombinant proteins in *E. coli* Rosetta. Lanes 1 and 3: cell supernatants of the sonicated pCold I vector and pCold-VP1 induced by IPTG; Lanes 2 and 4: precipitate of the sonicated pCold I vector and pCold-VP1 induced by IPTG. M: protein marker.

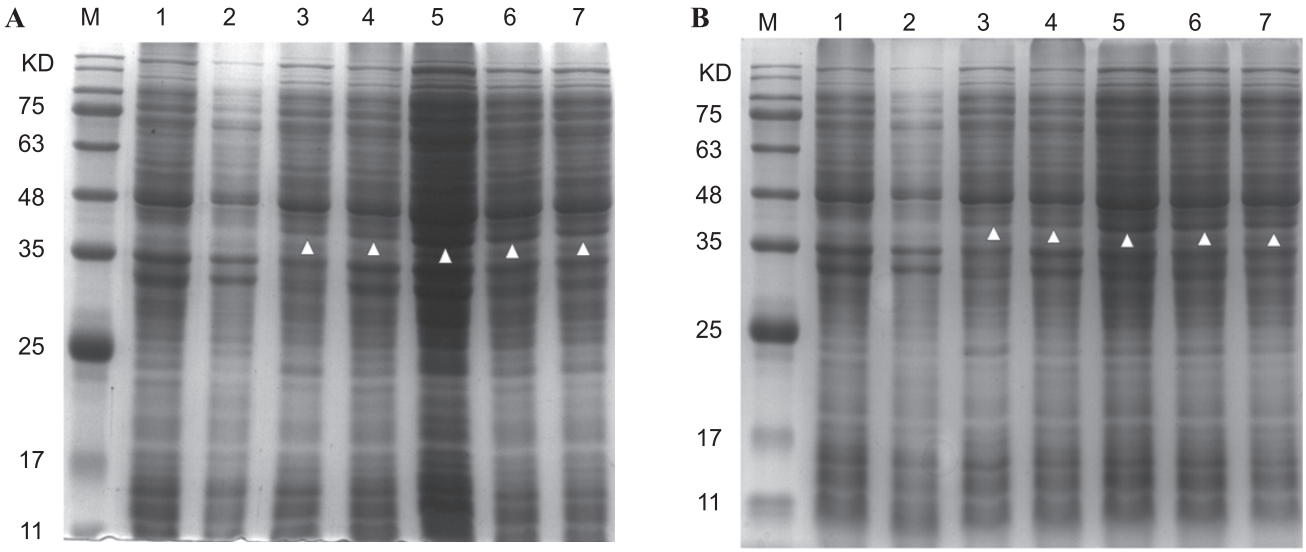

Fig. 3. Optimization of expression conditions. (A) Lane 1: precipitate of sonicated pCold vector transfection into *E. coli* Rosetta and induced by IPTG for 24 h; Lanes 2-7: precipitate of sonicated pCold-VP1 induced for 0 h, 8 h, 16 h, 24 h, 32 h and 40 h at 15°C by IPTG. (B) Lane 1: precipitate of sonicated pCold I vector induced by 0.6 mM IPTG for 24 h at 15°C; Lanes 2-7: precipitate of sonicated pCold-VP1 induction with IPTG for 0 mM, 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM and 1 mM, respectively. M: protein marker.

IPTG. The cell supernatant and cell pellet were analyzed after sonication by SDS-PAGE, and the results indicated that protein was not found in the cell supernatant and pCold I vector, while the recombinant plasmid pCold-VP1 was expressed in the culture cell pellet with the molecular weight at about 38 kDa, in accordance with the theoretical values (Fig. 2). After optimization of different induced time and concentrations of IPTG, we found that SV-A VP1 protein level was highest at 24 h (Fig. 3A) and with 0.6 mM ITPG (Fig. 3B). Therefore, a 0.6 mM concentration of IPTG in Rosetta strain for 24 h was considered to be the optimal condition for SV-A VP1 expression.

Purification of recombinant protein

Since SV-A 1D was expressed mainly in insoluble form, the inclusion body was dissolved in 8M urea. The supernatants of whole cell lysates were then used for protein purification. The protein was then purified

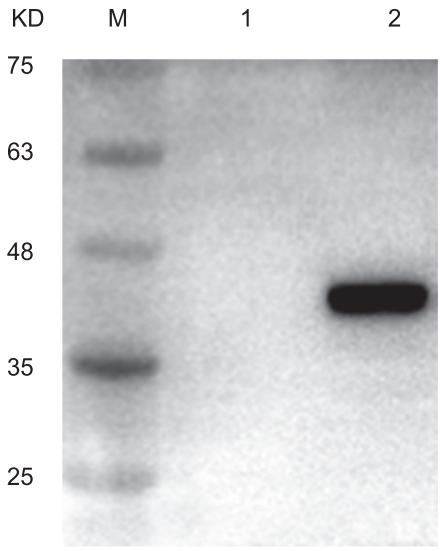

Fig. 4. Western blot analysis of SV-A VP1 protein. M: Protein marker; Lanes 1-2: Expression of purified SV-A VP1 protein incubated with negative and positive mouse serum against SV-A VP1, respectively.

with Ni-NTA Resin and eluted using various concentrations of imidazole. Upon purification, one clear protein band with molecular weight about 38 kDa was observed. The concentration of the purified His-tagged SV-A VP1 protein was 158 ng/mL.

Antiserum titer determination by ELISA

After the BALB/c mice were immunized three times with the purified SV-A VP1 protein, the antiserum was collected and the final titer of antiserum was detected by ELISA. The mean OD450 nm of the negative control was 0.1453 ± 0.031. The dilution ratio of serum ranged from 1:400 to 1:8,192,000. The ratio of positive absorbance value of antiserum to negative serum was 2.37 (1:2,048,000) and 1.52 (1:4,096,000). Therefore, the antiserum titer was determined to be 1: 2,048,000.

Western blot analysis and IFA

To evaluate the specificity of the purified anti-SV-A VP1 antiserum, western blot analysis and IFA were performed. Western blot analysis showed that anti-SV-A VP1 antiserum could recognize the *E.coli* expressed recombinant SV-A VP1 protein with a single band at about 38 kDa (Fig. 4). The immunoblotting data were further verified by IFA. SV-A infected PK-15 cells did not show immunofluorescence using negative serum (Fig. 5A), which suggested that mouse negative serum failed to react with SV-A infected PK-15 cells. However, strong green immunofluorescence was observed in PK-15 cells infected with SV-A using anti-SV-A VP1 polyclone antibody (Fig. 5B), indicating that mice serum of recombinant SV-A VP1 was able to recognize the native SV-A VP1 protein in PK-15 cells infected with SV-A and we had successfully obtained a mouse anti-SV-A VP1 polyclonal antibody which could identify SV-A.

Discussion

It has been reported that structural proteins play significant roles in viral functions including virus replication, production, fitness and pathogenicity (Fernandez-Sainz et al. 2014, Hussmann et al. 2014). However, studies on SV-A VP1 structural protein are rare. In this study, expression conditions of SV-A VP1 gene in *E.coli* Rosetta strains were explored and polyclonal antibody against VP1 was generated. Western blot analysis demonstrated that SV-A VP1 protein was specifically recognized by anti-SV-A VP1 positive serum, and IFA revealed that anti-SV-A VP1 positive serum could recognize the native SV-A VP1 protein infected with SV-A. These assays suggested that the SV-A VP1 gene was expressed at high levels in *E. coli* and the SV-A VP1 protein had good immunogenicity.

Expression of a heterologous recombinant protein in *E. coli* has been widely accepted for researchers because of its lower cost, ease of production and convenience. It has been proved very effective for recombinant capsid proteins, including Bluetongue virus VP2 (Zhou et al. 2017), and Chinese sacbrood virus VP1, VP2, and VP3 (Fei et al. 2015). In the present study, using this system, the SV-A VP1 was expressed in *E. coli* Rosetta. SDS-PAGE revealed that the expected size of the protein bands was found from cell lysates. The expected size of the protein bands was also found when detected by western blot analysis using mouse anti-SV-A VP1 as the primary antibody.

To optimize the *E. coli* expression system, more investigations should be performed to detect potential methods for obtaining higher protein production. A previous study has demonstrated that using a buffer containing 1.0 M NaCl followed by resolubilization using high pressure could obtain more human papillomavirus type 1 L1 protein from the bacterial pellet (Li et al. 1997). Additionally, by optimizing various rare amino acid codons, a higher level of pigeon circovirus capsid protein was obtained (Lai et al. 2014). In our study, different concentrations of IPTG and different induction times were used to optimize the expression levels, and a 0.6 mM concentration of IPTG in Rosetta strain for 24 h was considered as optimal expression conditions for the SV-A pCold- VP1 gene.

Many researchers chose the prokaryotic expression vectors pET32a (+) for producing the recombinant protein in *E. coli*, as it has a high stringency T7 lac pro-

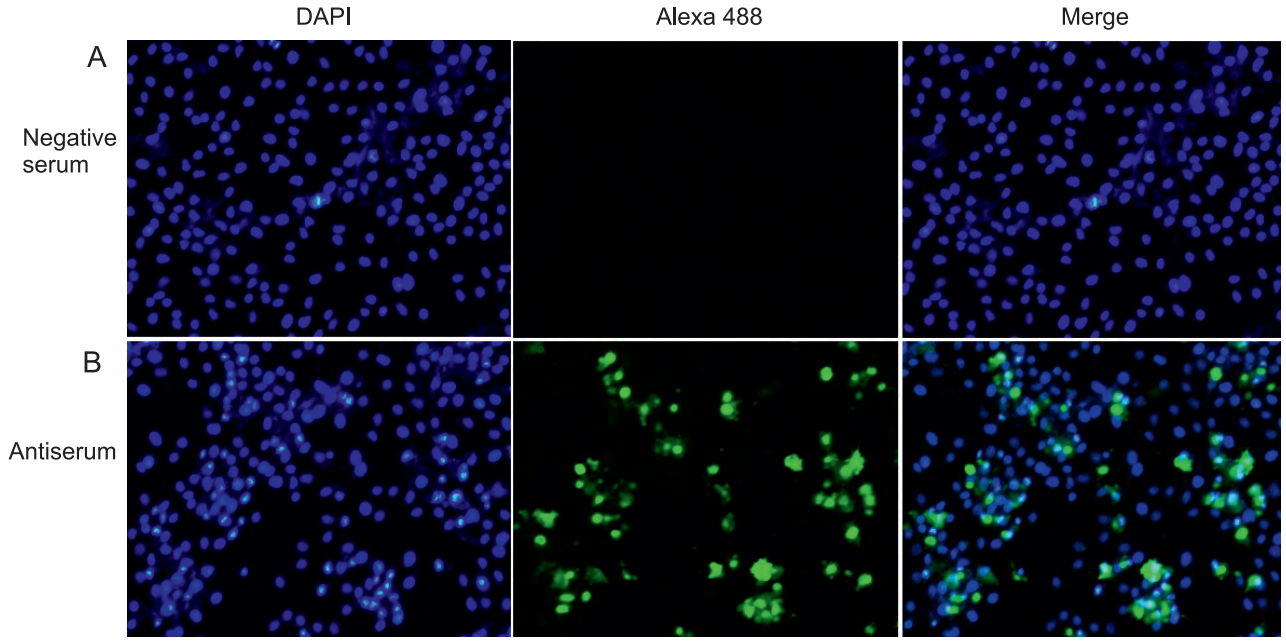

Fig. 5. PK-15 cells infected with SV-A virus (MOI = 1) for 30 h and analyzed by indirect immunofluorescence assay using mouse negative serum (A) or mouse anti-SV-A VP1 polyclonal antibody (B). DAPI: blue fluorescence; Alexa Fluor® 488: green fluorescence. ×200

moter and 6×His-tag (Chandrasekharaiah et al. 2011, Wang et al. 2015). However, in our study, we successfully produced SV-A VP1 in *E. coli* by using a cold shock expression system, pCold I vector. This contains cold shock protein A promoter, contributing to recombinant protein expression at lower incubation temperatures (15°C). Low-temperature induction renders not only expression of target proteins at high yields but also high purity, and increases the solubility of the protein. Using pCold vectors, many heterologous proteins, including norovirus virus (Huo et al. 2017) and human interleukin-1β (Zhu et al. 2015) have been expressed and obtained high yields.

Previous studies have found that monoclonal antibodies are specific to different VP1 protein domains (Hardy et al. 1996, Yoda et al. 2001). Monoclonal antibodies could recognize conformational epitopes on the N- or C-terminus of the capsid protein (Hardy et al. 1996). Yoda *et al.* (Yoda et al. 2001) have indicated that the N-terminal domain contains more antigenic epitopes, and thus is more immunodominant than the C-terminal region. However, the findings of Hardy et al. (Hardy et al. 1996) have revealed that the C-terminus encompasses the more immunodominant epitope protein. Broadly reactive monoclonal antibodies of SV-A could be used for developing a rapid diagnostic detection of SV-A. In addition, if SV-A-specific monoclonal antibodies contain common epitopes among all SV-A, it would be possible for the development of a broadly reactive ELISA to detect SV-A antigens. In this study, we produced a polyclonal antibody of mouse anti-SV-A VP1. Using this antibody, an obvious single band was found by western blot, and strong green immunofluorescence was observed in PK-15 cells infected with SV-A by IFA. However, cross-reactivity with other SV-A or other picornaviruses was not confirmed. More experiments may be needed to confirm cross-reactivity with other SV-A genogroups.

In conclusion, we successfully expressed SV-A VP1 protein and optimized the expression conditions, which paves the way for large-scale efficient production of SV-A VP1 protein. In addition, we purified SV-A VP1 protein and generated recombinant SV-A VP1 antigens, which were able to properly generate antibodies specific to SV-A VP1 proteins. We also demonstrated that self-prepared mouse anti-SV-A VP1 antibodies could recognize the native SV-A VP1 protein in PK-15 cells infected with SV-A, which may have the potential to allow the development of a serodiagnostic kit which could be used to detect SV-A infection in pigs.

Acknowledgements

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