Generation and characterization of a potentially applicable Vero cell line constitutively expressing the Schmallerberg virus nucleocapsid protein

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Abstract Schmallerberg virus (SBV) is a *Culicoides*-transmitted orthobunyavirus that poses a threat to susceptible livestock species such as cattle, sheep and goats. The nucleocapsid (N) protein of SBV is an ideal diagnostic antigen for the detection of viral infection. In this study, a stable Vero cell line, Vero-EGFP-SBV-N, constitutively expressing the SBV-N protein was established using a lentivirus system combined with puromycin selection. This cell line spontaneously emitted green fluorescent signals distributed throughout the cytoplasm, in which the expression of SBV-N fusion protein was confirmed by western blot analysis. The expression of SBV-N protein in Vero-EGFP-SBV-N cells was stable for more than fifty passages without puromycin pressure. The SBV-N fusion protein contained both an *N*-terminal enhanced green fluorescent protein (EGFP) tag and a *C*-terminal hexa-histidine ($6 \times \text{His}$) tag, by which the N protein was successfully purified using

Ni-NTA affinity chromatography. The cell line was further demonstrated to be reactive with SBV antisera and an anti-SBV monoclonal antibody in indirect immunofluorescence assays. Taken together, our results demonstrate that the Vero-EGFP-SBV-N cell line has potential for application in the serological diagnosis of SBV infection.

Keywords Schmallerberg virus · Nucleocapsid protein · Vero cells · Lentivirus transduction · Protein purification · SBV bovine antisera

Introduction

Schmallerberg virus (SBV) is a *Culicoides*-transmitted causative agent that was first identified in Germany in late 2011 (De Regge et al. 2014; Hoffmann et al. 2012; Rasmussen et al. 2012; Veronesi et al. 2013). It mainly infects ruminants such as cattle, sheep and goats, and while symptoms include fever, diarrhea and reduced milk production, infection can also result in congenital malformations and stillbirths (Doceul et al. 2013; Helmer et al. 2013; Hoffmann et al. 2012). Schmallerberg virus is classified in the genus *Orthobunyavirus* within the family *Bunyaviridae* (Hoffmann et al. 2012). The virus is enveloped and has a single-stranded negative-sense tripartite RNA genome consisting of large (L), medium (M) and small (S) segments. The S segment is relatively conserved

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and encodes two proteins in overlapping ORFs, the nucleocapsid (N) protein and a small nonstructural protein (Doceul et al. 2013). The N protein has a predicted molecular weight of about 26 kDa consisting of 233 amino acids (Doceul et al. 2013). It is the most abundant viral protein in infected cells and is highly immunogenic in infected animals, enabling it to elicit an early immune response soon after the onset of infection. Thus, these properties make the N protein a promising candidate for the detection of SBV infection, at both molecular and serological levels (Bilk et al. 2012; Bréard et al. 2013).

In recent years, mammalian cells have become a powerful tool for the production of recombinant proteins, especially those needing post-translational modifications (Bandaranayake and Almo 2014; Büssow 2015; Kantardjieff and Zhou 2014). A variety of mammalian cell lines stably expressing exogenous genes were successfully generated using lentiviral vectors (Liu et al. 2016; Phanthanawiboon et al. 2014; Zhang et al. 2015), which serve as efficient vehicles for transducing genes of interest into both dividing and nondividing cells, thereby achieving long-term stable expression of the transgenes (Nasri et al. 2014).

Highly permissive to SBV infection, Vero cells are successfully used to isolate, propagate and titrate SBV (Hulst et al. 2013; Mansfield et al. 2013; van der Heijden et al. 2013). In the present study, we generated a Vero cell line constitutively expressing the SBV-N protein using a lentiviral gene delivery system, and assessed its potential application in SBV diagnosis.

Materials and methods

Lentiviral vectors

Lentiviral transfer vector pLV-EGFP-C (Fig. 1a), packaging vector pHelper1.0 (Fig. 1b), and envelope vector pHelper2.0 (Fig. 1c) were purchased from Inovogen Tech. Co. Ltd (Beijing, China). The pLV-EGFP-C vector contains two eukaryotic promoters. One is the phosphoglycerate kinase promoter, which drives expression of the puromycin selectable marker. The other is the cytomegalovirus promoter, which drives expression of the EGFP reporter gene and the

foreign gene of interest cloned into the multiple cloning site.

Monoclonal antibody (mAb)

A mAb (clone 2C8) raised against the SBV-N protein was prepared in our laboratory (Zhang et al. 2013). This mAb reacts well with both recombinant SBV-N proteins and SBV isolates and was used to characterize our novel cell line.

SBV RNA and sera samples

SBV RNA of BH80/11-4 isolate (GenBank accession Nos. HE649912–HE649914) and two reference antisera (R1, bovine serum; R4, ovine serum) against SBV were kindly provided by the Friedrich-Loeffler-Institut (Greifswald-Insel Riems, Germany). Bovine serum HB826, determined to be negative for SBV antibody using a commercial SBV antibody enzyme-linked immunosorbent assay (ELISA) kit (ID Screen® Schmallenberg virus Competition, IDvet Innovative Diagnostics, Grabels, France), was prepared in our laboratory. Briefly, blood samples were taken from the external jugular vein using BD vacutainer tubes (BD Franklin Lakes, NJ, USA). After centrifugation at 3000 rpm for 10 min, the upper clarified serum was aliquoted and stored at -20°C .

Plasmid construction

The full-length SBV-N gene was amplified by reverse transcription PCR (RT-PCR) using the SBV RNA as a template, and specific forward (5'-GGT GAA TTC ATG TCA AGC CAA TTC ATT TTT GAA GAT GTA CCA CAA CGG AAT GC-3'; *EcoRI* recognition site underlined) and reverse primers (5'-TAA GGA TCC TTA ATG ATG ATG GTG GTG ATG GAT GTT GAT ACC GAA TTG CTG CA-3'; *BamHI* recognition sequence underlined; italics indicate the 6 × His coding sequence). The introduced 6 × His coding sequence was used to generate a C-terminal His-tagged SBV-N fusion protein, which can be purified by Ni-NTA affinity chromatography (Bréard et al. 2013; Zhang et al. 2013). The resulting PCR fragment (Fig. 1d) was then cloned into vector pLV-EGFP-C

Fig. 1 Schematic diagrams of the three lentivirus vectors and construction strategy. **a** Lentivirus transfer vector pLV-EGFP-C. *LTR* long terminal repeat, *Psi* packaging signal, *RRE* rev response element, *cPPT* central polypurine tract, *P_{CMV IE}* cytomegalovirus-immediate early promoter, *EGFP* enhanced green fluorescence protein, *MCS* multiple cloning site, *P_{PGK}* phosphoglycerate kinase promoter, *Puro (R)* puromycin resistance gene, *WPRE* woodchuck hepatitis post-transcriptional regulatory element, *pUC ori* pUC origin of replication, *Amp (R)* ampicillin resistance gene. **b** Lentivirus packaging vector pHelper1.0. *CMV enhancer* cytomegalovirus enhancer, *CAG*

promoter chicken β -actin promoter, *RRE* rev response element, *rev*, regulator of expression of virion proteins *PolyA site* polyadenylation site, *SV40 ori* Simian virus 40 origin of replication, *pBR322 ori* pBR322 origin of replication, *Amp (R)* ampicillin resistance gene. **c** Lentivirus envelope vector pHelper2.0. *CMV promoter* cytomegalovirus promoter, *VSV-G* vesicular stomatitis virus G protein, *pBR322 ori* pBR322 origin of replication, *Amp (R)* ampicillin resistance gene. **d** The SBV-N gene with a C-terminal hexa-histidine (6 $\times$ His) tag was cloned into vector pLV-EGFP-C between *EcoRI* and *BamHI* restriction sites

between *EcoRI* and *BamHI* restriction sites (Fig. 1a) to generate recombinant plasmid pLV-EGFP-SBV-N.

Lentivirus production

HEK293T cells were cultured in 10-cm-diameter dishes in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS;

Gibco) at 37 °C in a 5% CO₂ humidified incubator. The plasmids pLV-EGFP-SBV-N (5 μ g), pHelper1.0 (3.75 μ g) and pHelper2.0 (1.25 μ g) were co-transfected into HEK293T cells using PolyFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Infectious lentiviruses were harvested at 48 h post-transfection, filtered through a 0.45- μ m filter and aliquoted for storage at -70 °C as lentiviral stocks. The lentivirus generated

with pLV-EGFP-C was used as an empty vector control.

Lentiviral transduction

Transduction of Vero cells with recombinant lentiviruses was performed as previously described (Zhang et al. 2015). Briefly, Vero cells were seeded at a density of approximately 5×10^4 cells/well in a 24-well cell culture plate with DMEM containing 10% FBS. The cells were cultured at 37 °C in a 5% CO₂ humidified incubator for about 24 h. When cells reached approximately 60% confluence, the medium was replaced with a 1:10 (v/v) lentivirus stock solution diluted with DMEM and polybrene (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 6 µg/mL. After 24 h incubation, the supernatant was removed, fresh DMEM containing 10% FBS was added, and the cells were cultured for another 24 h.

Puromycin selection

Before starting puromycin selection, the working concentration of puromycin for Vero cells was determined using a series of concentrations ranging from 1 to 10 µg/mL. The lowest concentration that killed 100% of nontransduced Vero cells in 3–4 days from the start of puromycin selection was chosen as the optimal concentration in the subsequent experiments. Forty-eight hours after transduction, Vero cells were trypsinized, split into 10-cm culture dishes (200–500 cells/dish) and cultured in DMEM containing 10% FBS and 3 µg/mL puromycin (Sigma-Aldrich). During the selection period, the medium containing puromycin was exchanged at two-day intervals for about three weeks. Puromycin resistant cell colonies were manually picked, subcloned and passaged.

Western blot

Total cellular protein samples were prepared using RIPA lysis buffer (Beyotime, Jiangsu, China) according to the manufacturer's instructions (Liang et al. 2016; Wang et al. 2014). Briefly, cells were rinsed with PBS (pH 7.4) and lysed on ice for 30 min in RIPA lysis buffer supplemented with protease inhibitor phenylmethanesulfonyl fluoride (Beyotime, Jiangsu, China). After centrifugation at

12,000 rpm for 30 min at 4 °C, the supernatants of cell lysates were retained and aliquoted at −20 °C. 10 µL of cell lysates (approximately 30 µg) per well were separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and the proteins were transferred onto polyvinylidene fluoride membranes. After blocking with 5% (w/v) nonfat dry milk overnight at 4 °C, the membranes were probed with either 2C8 (1:4000), anti-EGFP (1:1000; Zoonbio Biotechnology Co., Ltd., Nanjing, China), anti-His (1:1000; ZSGB-Bio Co., Ltd., Beijing, China) or anti-β-actin (1:1000; Sigma-Aldrich, St. Louis, MO, USA) mAbs as primary antibodies, followed by HRP-conjugated goat anti-mouse secondary antibody (1:8000). Blots were developed with an enhanced chemiluminescence detection kit and a medical X-ray processor (Kodak, Rochester, NY, USA). Images were acquired using an image scanner (Seiko Epson Corp., Nagano-ken, Japan). The grey value of target protein bands was analyzed using the Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA, USA).

Protein purification

Schmallenberg virus N fusion protein expressed in Vero-EGFP-SBV-N cells was purified under native conditions using Ni–NTA agarose according to the manufacturer's instructions (Qiagen 2003), with slight modifications (Zhang et al. 2015). Briefly, approximately 4.2×10^8 cells were collected and lysed with RIPA lysis buffer. The lysate was further sonicated at 75 W for six cycles (15 s each) on ice with 10-s cooling intervals between each cycle. Sonicated lysate was cleared by centrifugation at 10,000×g for 10 min at 4 °C, and the supernatant was filtered through a 0.22-µm filter. The filtrate was mixed with 2 mL Ni–NTA slurry by gently shaking at 4 °C for 2 h. The lysate–Ni–NTA mixture was loaded into a column, and thoroughly washed with 20-, 50-, and 100-mM imidazole step gradients in wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 0.05% Tween 20, pH 8.0). The bound SBV-N fusion protein was eluted four times with 0.5-mL elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluate was concentrated to 500 µL using a centrifugal filter concentrator, and protein concentration was determined by the Bradford assay.

Immunofluorescence assay (IFA)

Indirect IFA was performed on Vero cells using a previously described protocol (Zhang et al. 2015). Briefly, Vero cells grown on coverslips were counterstained with Hoechst 33342 and then fixed with pre-chilled 100% ethanol for 15 min. After thoroughly washing with PBS, cells were incubated with mAb 2C8 (1:1000), SBV antisera (1:100; Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany) or bovine SBV-antibody negative serum HB826 (1:100) for 1 h at 37 °C. Cells were then washed with PBS and incubated for 1 h with TRITC-conjugated goat anti-mouse (1:100; Sigma-Aldrich), rabbit anti-bovine IgG (1:100; ImmunoReagents, Raleigh, NC, USA) or donkey anti-sheep IgG (1:100; ImmunoReagents), respectively. Afterwards, the cells were rinsed and then mounted onto slides with anti-fade mounting medium (Beyotime). Cells were observed with a Leica confocal microscope (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

Data were presented as arithmetic mean $\pm$ standard deviation (SD). Statistical significance was analyzed by one-way analysis of variance (ANOVA) test using the GraphPad Prism Software (La Jolla, CA, USA), and values of $P < 0.05$ were considered statistically significant.

Results

Construction of recombinant lentiviral transfer plasmid

To facilitate protein purification, the coding sequence for a 6 $\times$ His tag was introduced into the 5' end of the reverse primer. After amplification by RT-PCR, the resulting amplicon was cloned downstream of the EGFP coding sequence of the lentiviral transfer vector, pLV-EGFP-C, between the *EcoRI* and *BamHI* restriction sites (Fig. 1a, d). The resulting recombinant plasmid, designated pLV-EGFP-SBV-N, was further analyzed by DNA sequencing. The results showed that the SBV-N gene was successfully fused in-frame with an N-terminal EGFP and a C-terminal 6 $\times$ His tag,

and the nucleotide sequence was correct (Supplemental Fig. S1).

Production of recombinant lentiviruses in HEK293T cells

After transfection, EGFP expression in HEK293T cells was monitored by fluorescence microscopy, with expression peaking at 48 h post-transfection (data not shown). At this time-point, recombinant lentiviruses were harvested, filtered and aliquoted as viral stocks. The lentiviruses generated with pLV-EGFP-SBV-N and pLV-EGFP-C were designated rLenti-EGFP-SBV-N and rLenti-EGFP, respectively.

Puromycin selection of Vero cell lines

After transduction of Vero cells with rLenti-EGFP-SBV-N, three Vero cell lines spontaneously emitting green fluorescent signals were obtained following puromycin selection. Western blot analysis confirmed the expression of SBV-N protein in these three cell lines (Fig. 2a). The cell line showing the highest expression level was designated Vero-EGFP-SBV-N and selected for further analysis (Fig. 2b), while the other two cell lines were designated Vero-EGFP-SBV-N (I) and Vero-EGFP-SBV-N (II), respectively. The signaling pattern of Vero-EGFP-SBV-N resembled that of Vero-EGFP, a control cell line generated by transducing Vero cells with rLenti-EGFP. Both Vero-EGFP-SBV-N and Vero-EGFP exhibited homogenous fluorescence distribution throughout the cytoplasm (Fig. 2c).

Analysis of SBV-N protein expression in Vero cell lines

Expression of SBV-N fusion protein in generated cell lines was determined by western blot analysis with β -actin as the internal control. As shown in Fig. 3a, a protein band of the expected size (52 kDa) was only detected in the Vero-EGFP-SBV-N cell line using mAb 2C8 as the primary antibody, but not in the empty vector control Vero-EGFP cell line or the non-transduced control Vero cells. Recognition of this protein by anti-EGFP and -His mAbs suggested that it was a fusion protein comprising SBV-N protein, EGFP and His tags. As expected, a 26-kDa protein band was detected in the Vero-EGFP cell line using

Fig. 2 Vero cell lines constitutively expressing SBV-N fusion protein. **a** Western blot analysis of SBV-N fusion protein expressed in the generated three Vero cell lines. β-Actin was used as the internal control. *Lane 1* Total cellular proteins of the Vero-EGFP-SBV-N (I) cell line, *Lane 2* Total cellular proteins of the Vero-EGFP-SBV-N (II) cell line, *Lane 3* Total cellular proteins of the Vero-EGFP-SBV-N cell line. The molecular

weights of the target proteins are indicated to the *left* in kDa. **b** Analysis of relative levels of SBV-N fusion protein expressed in the generated three Vero cell lines. Assays were performed in triplicate and data were expressed as mean $\pm$ SD (* P < 0.05; ** P < 0.01). **c** Fluorescence microscopy analysis of the generated Vero cell lines. Scale bars 50 μm

Fig. 3 Expression of SBV-N fusion protein in the generated Vero cell lines. **a** Western blot analysis of SBV-N fusion protein expressed in the generated Vero cell lines. β -Actin was used as the internal control. *Lane 1* Total cellular proteins of Vero-EGFP-SBV-N cell line, *Lane 2* Total cellular proteins of Vero-EGFP cell line (empty vector control), *Lane 3* Total cellular proteins of Vero cells (blank control). **b** and **c** Stability analysis

anti-EGFP mAb as the primary antibody, which corresponds to the theoretical molecular weight for the EGFP tag. Moreover, to assess the stability of the Vero-EGFP-SBV-N cell line, it was serially passaged for up to 50 passages in the absence of puromycin. Protein samples from cells at the first, 10th, 20th, 30th, 40th, and 50th passages were detected by western blot analysis using 2C8 and anti- β -actin mAbs as primary antibodies (Fig. 3b). The protein bands were quantitatively analyzed by grey value determination, and the data showed that there was no significant difference in the expression levels of SBV-N protein between cell passages ($P > 0.05$; Fig. 3c), showing that SBV-N fusion protein expression in the Vero-EGFP-SBV-N cell line was stable.

Purification of SBV-N protein from the Vero-EGFP-SBV-N cell line

To explore whether the C-terminal $6 \times$ His tag introduced via the reverse primer was effective for purification of SBV-N fusion protein from Vero-EGFP-SBV-N cells, Ni-NTA agarose was used to

of the generated Vero-EGFP-SBV-N cell line. Protein samples from cells at the 1st, 10th, 20th, 30th, 40th, and 50th passages in the absence of puromycin were detected by western blot analysis. Assays were performed in triplicate and data were expressed as mean $\pm$ SD ($^{\#} P > 0.05$, as compared to each other). The molecular weights of the target proteins are indicated to the left in kDa

purify this protein. As shown in Fig. 4a, a 52-kDa protein was detected after Coomassie brilliant blue staining, with a purity of about 90%. Its concentration was determined to be 588 μ g/mL, which is equivalent to a protein yield of 7 μ g per 10^7 Vero-EGFP-SBV-N cells. Western blot analysis demonstrated that the purified protein reacted not only with 2C8 but also with mouse anti-His and -EGFP mAbs (Fig. 4b), indicating the fusion of SBV-N protein with EGFP, His tags and co-expression in Vero-EGFP-SBV-N cells. Taken together, these results suggest that the Vero-EGFP-SBV-N cell line can serve as a sustainable source of recombinant SBV-N protein.

Reactivity of Vero-EGFP-SBV-N cell line with antibodies against SBV

To assess the reactivity of Vero-EGFP-SBV-N cell line with SBV antibodies, indirect IFA was performed using mAb 2C8 and bovine SBV antiserum R1 as primary antibodies. After immunological reaction, the red immunostaining signals corresponding to the SBV-N protein were highly co-localized with the

Fig. 4 Purification and identification of SBV-N fusion protein from the Vero-EGFP-SBV-N cell line. **a** SDS-PAGE analysis of the purified SBV-N fusion protein and total cellular proteins of the Vero-EGFP-SBV-N cell line. *Lane M* Protein molecular weight marker, *Lane 1* Purified SBV-N fusion protein, *Lane 2*

Total cellular proteins of the Vero-EGFP-SBV-N cell line. **b** Western blot analysis of the purified SBV-N fusion protein. *Lane M* Protein molecular weight marker, *Lane 1* Anti-EGFP mAb, *Lane 2* anti-SBV-N mAb, *Lane 3* anti-His mAb

green auto-fluorescent signals in Vero-EGFP-SBV-N cells (Fig. 5a, b), indicating this cell line reacts well with SBV-specific antibodies. Reactivity of this cell line with the ovine SBV antiserum R4 provided verification (data not shown). By contrast, this cell line did not react specifically with SBV antibody-negative serum (Fig. 5c). These results indicate a potential application for Vero-EGFP-SBV-N cell line in the serodiagnosis of SBV infection.

Discussion

As a newly emerged orthobunyavirus, SBV has posed a considerable threat to the susceptible livestock in Europe since 2011 (Doceul et al. 2013; Hoffmann et al. 2012). Development of specific diagnostic reagents and assays are important for controlling this emerging pathogen. Shortly after the initial outbreak

in Germany, a real-time quantitative RT-PCR (RT-qPCR) targeting the S segment of SBV genome was established to detect SBV infection (Bilk et al. 2012; Hoffmann et al. 2012). Besides, other RT-qPCRs that target the L and M segments of SBV genome, respectively, were also developed for SBV diagnosis (Fischer et al. 2013). Undoubtedly, these developed molecular methods have played an important role in SBV diagnosis. However, what needs to be emphasized is that SBV infection usually causes a very short duration of viremia lasting 3–5 days and a seroconversion occurring between days 7 and 14 post-infection in susceptible animals (Gubbins et al. 2014; Laloy et al. 2015; Wernike et al. 2013). This means that the window period during which SBV genome can be detected by RT-qPCR is very short. Moreover, although the seroconverted animals were demonstrated to have a relatively lower risk of transmitting SBV (Elbers et al. 2014; Poskin et al.

Fig. 5 Confocal microscopy analysis of the reactivity of the Vero-EGFP-SBV-N cell line with antibodies against SBV. **a** Reactivity of Vero-EGFP-SBV-N cells with mAb 2C8. **b** and **c** Reactivity of Vero-EGFP-SBV-N cells with bovine SBV

antisera R1 or SBV-antibody negative serum HB826. *Green*, auto-fluorescence; *Blue*, cell nuclei; *Red*, immunostaining fluorescence corresponding to the SBV-N protein. Scale bars 25 μm. (Color figure online)

2015; Rodríguez-Prieto et al. 2016), several studies have shown that the semen excreted by a few seropositive bulls contained infectious SBV which could cause an acute infection in cattle and/or *IFNAR*^{-/-} mice through subcutaneous inoculation (Hoffmann et al. 2013; Ponsart et al. 2014; Schulz et al. 2014). What makes things worse is that the excretion of SBV in bovine semen was demonstrated to be in an intermittent manner (Hoffmann et al. 2013). Thus, other diagnostic methods especially serological assays, such as ELISA and IFA, need to be developed for the detection of SBV antibodies. Ample evidence has shown that SBV-specific antibodies, which

are reliable serodiagnostic markers for SBV infection, can persist in the infected animals for up to 24 months (Elbers et al. 2014; Wernike et al. 2015). Currently, both indirect and competitive ELISA kits detecting SBV antibodies are commercially available (Bréard et al. 2013; Molenaar et al. 2015); however, the high cost of these kits is a potential drawback which may limit their wide use in screening for SBV infection. Therefore, cost-effective diagnostic methods still need to be developed. As a commonly used serological method for the detection of serum antibodies, IFA was also established to detect SBV infection (Bréard et al. 2013); however, conventional

IFA requires an antigen matrix which was made by infecting permissive cells with live SBV. Handling infectious SBV in non-endemic countries may pose a potential threat to the livestock. Therefore, a safe, potent, cost-effective, and noninfectious diagnostic antigen is needed for detecting SBV infection.

Lentiviral vectors have been shown to be excellent tools for generating mammalian cell lines stably expressing exogenous genes (Liu et al. 2016; Phanthanawiboon et al. 2014; Zhang et al. 2015). In the present study, we successfully generated a Vero cell line constitutively expressing the SBV-N protein using a lentivector-mediated gene transfer system in combination with puromycin selection. As an intracellular reporter, the constitutively co-expressed EGFP provides a convenient and real time marker for monitoring the expression of exogenous genes in generated cell lines. The generated Vero-EGFP-SBV-N cell line was demonstrated to be able to spontaneously emit green fluorescent signals distributed homogeneously throughout the entire cytoplasm; however, its signaling pattern was quite different from that of our previously reported BHK-EGFP-SBV-N cell line (Zhang et al. 2015), which exhibited a granular fluorescent pattern in the cytoplasm. To find a possible explanation for this discrepancy, we determined the relative expression levels of SBV-N protein in the two cell lines and found that Vero-EGFP-SBV-N exhibited a relatively lower expression level than its counterpart BHK-21-EGFP-SBV-N. Of the five BHK-21 and three Vero cell lines we generated, BHK-21-EGFP-SBV-N exhibited the highest expression level of SBV-N protein (Supplemental Fig. S2). Accordingly, we speculate that only when the cytoplasmic concentration of SBV-N protein reached a certain level could the cell line exhibit cytoplasmic protein aggregates, thus presenting a punctate fluorescent signaling pattern. Although the two cell lines possess a different signaling pattern, they have their own advantages. The Vero-EGFP-SBV-N cell line grows slightly faster than BHK-21-EGFP-SBV-N. By contrast, BHK-21-EGFP-SBV-N cell line has a relatively higher expression level of SBV-N fusion protein than Vero-EGFP-SBV-N. Also, BHK-21-EGFP-SBV-N cell line presents punctate fluorescent signals that could be more easily observed in IFA test.

In order to assess whether the Vero-EGFP-SBV-N cell line is potentially applicable for the serological diagnosis of SBV infection, we analyzed its reactivity with the mAb 2C8 and SBV antisera and found it could

be used as a safe antigen matrix for immunofluorescence detection of SBV-specific antibodies; however, further investigation is necessary to evaluate the sensitivity and specificity of this cell line-based indirect IFA by using more field sera samples. Moreover, we determined the stability of Vero-EGFP-SBV-N cell line by continuous cell passage and proved that SBV-N protein expression in this cell line was stable for more than fifty passages even without puromycin selective pressure, indicating the cell line has good stability. Since a 6 × His tag was intentionally introduced into the C-terminus of SBV-N fusion protein, we tried to purify SBV-N fusion protein from Vero-EGFP-SBV-N cells using Ni-NTA affinity chromatography. The introduced C-terminal His tag was demonstrated to be effective for purification of SBV-N protein from Vero-EGFP-SBV-N cells. Since Vero cells grow fast and can be cultured in suspension (Paillet et al. 2009), the Vero-EGFP-SBV-N cell line provides a sustainable source for the large-scale production of recombinant SBV-N protein. It is noteworthy that although the recombinant SBV-N fusion protein carries a relatively large N-terminal EGFP tag, it reacted well with the mAb 2C8 and SBV antisera in indirect IFA and western blot analysis. This reveals that the EGFP tag did not affect the reactivity of the SBV-N fusion protein with SBV-specific antibodies. Taken together, our results suggest that the generated Vero-EGFP-SBV-N cell line holds promise for use in the serodiagnosis of Schmallenberg disease.

Conclusion

A stable Vero cell line, Vero-EGFP-SBV-N, constitutively expressing the SBV-N protein was established, which spontaneously emitted green fluorescent signals distributed throughout the cytoplasm. This cell line not only provides a sustainable source of recombinant SBV-N protein, but also shows potential for application in the serodiagnosis of SBV infection.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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