Molecular and Cellular Probes 29 (2015) 244–253

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Preparation and characterization of a stable BHK-21 cell line constitutively expressing the Schmallenberg virus nucleocapsid protein

Yongning Zhang, Shaoqiang Wu, Shanshan Song, Jizhou Lv, Chunyan Feng, Xiangmei Lin*

Institute of Animal Quarantine, Chinese Academy of Inspection and Quarantine, Beijing 100029, China

ARTICLE INFO

Article history:
Received 6 March 2015
Received in revised form
9 May 2015
Accepted 22 May 2015
Available online 23 May 2015

Keywords:
Schmallenberg virus (SBV)
Nucleocapsid (N) protein
BHK-21 cells
Lentivirus vector
SBV antisera

ABSTRACT

Schmallenberg virus (SBV) is a newly emerged orthobunyavirus that predominantly infects livestock such as cattle, sheep, and goats. Its nucleocapsid (N) protein is an ideal target antigen for SBV diagnosis. In this study, a stable BHK-21 cell line, BHK-21-EGFP-SBV-N, constitutively expressing the SBV N protein was obtained using a lentivector-mediated gene transfer system combined with puromycin selection. To facilitate the purification of recombinant SBV N protein, the coding sequence for a hexa-histidine tag was introduced into the C-terminus of the SBV N gene during construction of the recombinant lentivirus vector pLV-EGFP-SBV-N. The BHK-21-EGFP-SBV-N cell line was demonstrated to spontaneously emit strong enhanced green fluorescent protein (EGFP) signals that exhibited a discrete punctate distribution throughout the cytoplasm. SBV N mRNA and protein expression in this cell line were detected by real-time RT-PCR and western blot, respectively. The expressed recombinant SBV N protein carried an N-terminal EGFP tag, and was successfully purified using Ni–NTA agarose by means of its C-terminal His tag. The purified SBV N protein could be recognized by SBV antisera and an anti-SBV monoclonal antibody (mAb) 2C8 in an indirect enzyme-linked immunosorbent assay and western blot analyses. Indirect immunofluorescence assays further demonstrated that the stable cell line reacts with SBV antisera and mAb 2C8. These results suggest that the generated cell line has the potential to be used in the serological diagnosis of SBV.

1. Introduction

Schmallenberg disease is an arboviral disease caused by Schmallenberg virus (SBV), which was first identified in Germany in late 2011 [1]. The disease was first observed in domestic ruminants such as cattle, sheep and goats [1–3]. Following these initial observations, infections were also confirmed in a variety of deer, bison, alpacas, mouflons, buffaloes, dogs, pigs, wild boars, NIH-Swiss mice, and type I interferon receptor knock-out mice [4–7]. The main clinical symptoms are transient fever, decreased milk production, inappetence and diarrhea in adult ruminants [1,5]. Infection of pregnant animals with SBV during a critical period of gestation can result in abortions, fetal malformations or stillbirths [5,8,9]. Certain *Culicoides* biting midges have been demonstrated to be the main vector for SBV transmission [10,11]. SBV can also be vertically transmitted from mother to fetus through the placenta [8,12–14]. Other routes of transmission, such as horizontal transmission by direct contact, have yet to be demonstrated [3,15].

SBV is classified as a member of the Simbu serogroup in the genus *Orthobunyavirus* within the family *Bunyaviridae* [1]. Similar to other bunyaviruses, SBV has a single-stranded negative-sense tripartite RNA genome consisting of large (L), medium (M) and small (S) segments. The M segment encodes two surface glycoproteins (Gn and Gc) and a non-structural protein NSm. The S

Abbreviations: SBV, Schmallenberg virus; N, nucleocapsid; EGFP, enhanced green fluorescent protein; mAb, monoclonal antibody; IFA, indirect immunofluorescence assay; His, histidine; HRP, horseradish peroxidase; IgG, immunoglobulin G; TRITC, tetramethylrhodamine isothiocyanate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Ni–NTA, nickel nitrilotriacetic acid; RT-PCR, reverse transcription polymerase chain reaction; rRT-PCR, real-time RT-PCR; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween 20; ELISA, enzyme-linked immunosorbent assay; Ct, cycle threshold; MCS, multiple cloning sites.

* Corresponding author. Institute of Animal Quarantine, Chinese Academy of Inspection and Quarantine, Building No. 241 Huixinli, Chaoyang District, Beijing 100029, China.

E-mail addresses: ynzhang-2008@163.com (Y. Zhang), linxm@caiq.gov.cn (X. Lin).

http://dx.doi.org/10.1016/j.mcp.2015.05.010
0890-8508/

segment encodes the nucleocapsid (N) protein and a nonstructural protein NSs [5,16]. The N protein consists of 233 amino acids and has a predicted molecular weight of approximately 26 kDa [16,17].

A previous study demonstrated that SBV grows well in several cell lines derived from various animal species and humans, and causes significant cytopathic effects in most of the tested cell lines [7]. To date, BHK-21 and Vero cells have been successfully used for the isolation, cultivation and indirect immunofluorescence assay (IFA) of SBV [1,18]. Given its abundance in the virion and in infected cells, SBV N protein has been widely used for the molecular and serological diagnosis of SBV infection [18,19]. In the present study, we attempted to generate a stable BHK-21 cell line that constitutively expresses the SBV N protein using a lentivirus system and characterize the resulting cell line.

2. Materials and methods

2.1. Antibodies, vectors, reagents and kits

A monoclonal antibody (mAb) raised against the SBV N protein (clone 2C8; IgG2α, kappa isotype) and a prokaryotic SBV N protein carrying both an N- and a C-terminal histidine (His) tag (designated His-SBV-N) were prepared in our laboratory [20]. Mouse anti-enhanced green fluorescent protein (EGFP; ABM-0005) and anti-His (ABM-0008) mAbs were purchased from Zoonbio Biotechnology Co., Ltd (Nanjing, China). Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG; A4416), tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (T5393), mouse anti-β-actin mAb (A1978), HRP-conjugated rabbit anti-bovine IgG (A5295) and HRP-conjugated donkey anti-sheep IgG (A3415) were purchased from Sigma—Aldrich (St. Louis, MO, USA). TRITC-conjugated rabbit anti-bovine IgG (RbxBo-003-DRHOX) and donkey anti-sheep IgG (DkxSh-003-FRHOX) were obtained from ImmunoReagents, Inc. (Raleigh, NC, USA).

The three lentivirus vectors (transfer vector, pLV-EGFP-C; packaging vector, pHelper1.0; and envelope vector, pHelper2.0) were purchased from Inovogen Tech. Co. Ltd (Beijing, China). The vector information is available on the Inovogen website (http://www.inovogen.com/lentivirus/lentivirusvector).

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), horse serum and AgPath-ID™ one-step RT-PCR kits (AM1005) were purchased from Life Technologies (Grand Island, NY, USA). Puromycin dihydrochloride (P7255) and polybrene (H9268) were purchased from Sigma—Aldrich. RIPA lysis buffer (P0013B) was purchased from Beyotime Institute of Biotechnology (Haimen, China). Plasmid plus maxi kits (12963), RNase-free DNase set (79254), nickel nitrilotriacetic acid (Ni—NTA) agarose (30210), polypropylene columns (34924) and PolyFect transfection reagent (301105) were purchased from Qiagen (Hilden, Germany). Gel extraction kits were purchased from Omega Bio-Tek (Norcross, GA, USA). Western luminescent detection kits were purchased from Vigorous Biotechnology Beijing Co., Ltd (Beijing, China). Restriction enzymes *EcoR*I and *BamH*I, DNA ligation kits, and one-step RNA PCR kits (AMV) were purchased from TaKaRa Biotechnology (Dalian, China). PageRuler™ prestained protein ladders (26616) were purchased from Thermo Scientific (Rockford, IL, USA).

2.2. SBV RNA and SBV antibody-positive and -negative serum samples

An RNA sample of the SBV BH80/11-4 isolate (GenBank accession Nos. HE649912—HE649914) and SBV antisera (R1, bovine serum; R4, ovine serum) were provided by Dr. Bernd Hoffmann and Dr. Martin Beer, respectively (Friedrich-Loeffler-Institut [FLI], Insel Riems, Germany). An SBV antibody-negative bovine serum HB826, determined with a commercial SBV antibody ELISA kit (ID Screen® Schmallenberg virus Competition, IDvet, France), was prepared in our laboratory.

2.3. Cell lines and cell culture

293T cells and BHK-21 cells were cultured in DMEM supplemented with 10% FBS. Cells were cultured at 37 °C/5% CO_2 in a humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Generation of recombinant lentivirus transfer vector

The full-length SBV N gene was amplified by reverse transcription polymerase chain reaction (RT-PCR) using 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′; *EcoR*I 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′; *BamH*I recognition sequence underlined; italics indicate the 6× His coding sequence). To facilitate the purification of SBV N protein, the coding sequence for a 6× His tag was introduced into the 5′ end of the reverse primer, resulting in a 6× His tag at the C-terminus of the recombinant SBV N protein. All PCRs were performed in a 50-μl volume using a commercial RT-PCR kit (TaKaRa) comprising 10× one step RNA PCR buffer (5 μl), 25 mM $MgCl_2$ (10 μl), 10 mM dNTPs (5 μl), 40 U/μl RNase inhibitor (1 μl), 5 U/μl AMV reverse transcriptase XL (1 μl), 5 U/μl AMV-optimized *Taq* (1 μl), 10 μM forward primer (2 μl), 10 μM reverse primer (2 μl), SBV RNA template (4 μl), and RNase-free water (19 μl). Thermal cycling conditions involved an initial 30-min incubation at 50 °C, then 94 °C for 2 min, followed by 30 cycles of amplification (94 °C for 30 s, 62 °C for 30 s, and 72 °C for 45 s). Amplicons were separated and extracted from 1% (w/v) agarose gels using a DNA gel extraction kit (Omega). The recovered amplicons and pLV-EGFP-C vector were simultaneously digested with *EcoR*I and *BamH*I, respectively. After another gel extraction step, the two recovered fragments were ligated with T4 ligase to yield a recombinant lentivirus transfer vector, designated pLV-EGFP-SBV-N.

2.5. Production of recombinant lentiviruses and determination of the virus titer

293T cells were seeded in 10-cm culture dishes at a density of 5×10^6 cells/dish, and incubated at 37 °C/5% CO_2 until cultures grew to approximately 90% confluence. The pLV-EGFP-SBV-N plasmid and the two helper plasmids, pHelper1.0 and pHelper2.0, were extracted from transformed *Escherichia coli* DH5α with a plasmid extraction kit (Qiagen). We prepared 500 μl of solution A by adding 5 μg of pLV-EGFP-SBV-N plasmid, 3.75 μg of pHelper1.0 and 1.25 μg pHelper2.0 into an appropriate volume of serum-free DMEM. We prepared 500 μl of solution B by adding 20 μl of PolyFect transfection reagent (Qiagen) into 480 μl of serum-free DMEM. After thorough mixing, solution B was slowly added to solution A and mixed, incubated for 5 min at room temperature, and then added drop-wise to the 293T cells cultured in the 10-cm dishes. Cells were allowed to incubate at 37 °C/5% CO_2 for 6 h and then the mixture was replaced with 10 ml of fresh DMEM containing 10% FBS. After an additional 20 h of culturing, the medium was exchanged with 10 ml of fresh DMEM containing 10% FBS and 1 mM pyruvic acid and maintained at 37 °C/5% CO_2. Infectious lentiviruses were harvested at 48 h post-transfection by collecting culture supernatants, centrifuging them (4000× *g*, 10 min, 4 °C) to remove cellular debris, and filtering through a 0.45-μm filter. Lentivirus