

Production of CCHF Virus-Like Particle by a Baculovirus-Insect Cell Expression System*

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Abstract: Crimean-Congo Haemorrhagic Fever Virus (CCHFV) is a tick-born virus of the *Nairovirus* genus within the *Bunyaviridae* family, which is widespread and causes high fatality. The nucleocapsid of CCHFV is comprised of N proteins that are encoded by the S segment. In this research, the N protein of CCHFV was expressed in insect cells using a recombinant baculovirus. Under an electron microscope, Virus-Like Particles (VLPs) with various size and morphology were observed in cytoplasmic vesicles in the infected cells. Sucrose-gradient purification of the cell lysate indicated that the VLPs were mainly located in the upper fraction after ultracentrifugation, which was confirmed by Western blot analysis and immuno-electron microscopy (IEM).

Key words: Crimean-Congo hemorrhagic fever virus (CCHFV); Virus-Like Particle (VLP); Nucleocapsid (N) protein

Crimean-Congo Haemorrhagic Fever virus (CCHFV) is a tick-born virus of the *Nairovirus* genus within the *Bunyaviridae* family. It is a virus with three segments of single-stranded, negative RNAs [7, 8, 32]. The three segments are named S (small, ~1.7 kb), M (middle, ~5.7 kb) and L (large, ~12 kb), principally encoding

nucleocapsid protein (N protein, NP), glycoprotein and RNA-dependent RNA polymerase (RdRp), respectively [5, 7]. CCHFV has been found in more than 30 countries in Africa, Europe and Asia [23]. In China, it was first found in Xinjiang province in 1965 and outbreaks have been reported from time to time in Xinjiang [2, 21]. Apart from Xinjiang, CCHFV antibody positives were reported in animals or humans from several other provinces such as Yunnan, Qinghai and Sichuan [26, 34] and sporadic CCHF outbreaks have been recently reported in Turkey and Iran [3, 6, 22]. A CCHF outbreak poses a threat to public health because of the high mortality rate as well as the difficulties in treatment and prevention. 30-50% mortality is common and rates as high as 80% have also been reported [3, 13, 14, 32],

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mortality is related to the transmission routes and viral dose [3, 14, 32]. To date, the only known and used drug to treat CCHF is ribavirin [9] and there is no effective vaccine available against CCHFV [9].

Viral envelope proteins are the first-choice for vaccine design. However, they are more prone to mutation to evade the host immune system [11]. The nucleocapsid (N) proteins are normally comparatively abundant and conserved in viruses, therefore N proteins have been widely tested as protective immunogens. The N protein of the feline infectious peritonitis virus (FIPV) was found to induce protective immunity in cats [30]; the N protein of Influenza virus, Ebola virus and avian coronavirus can elicit a strong cellular immune response [12, 19, 33]. These results indicate the nucleocapsid protein is a promising candidate for vaccine development [17, 29]. The N protein of CCHFV is highly immunogenic [25, 31]. In this paper, the N protein of CCHFV was expressed by the baculovirus expression system, which is a candidate for protein overexpression and is extensively used for vaccine development. By Western blot and IEM analysis, it was shown that CCHFV N protein can automatically assemble into Virus-Like Particles (VLPs) when over expressed in insect cells.

MATERIALS AND METHODS

Insect cells and virus

Spodoptera frugiperda cell line Sf9 cells were cultured at 27 °C in Grace's Insect Medium (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS). AcMNPV bacmid bMON14272 (Invitrogen) used in the experiment was stored by our laboratory. The control virus *Ac-egfp* was previously constructed in our laboratory [27].

Construction of donor plasmids and Generation of recombinant bacmid

The S segment (GenBank accession no. FJ562093) of strain YL04057 CCHFV was kindly provided by Xinjiang CDC [31]. The complete NP ORF including the 5'-UTR and 3'-UTR was amplified using forward primer SF: 5'-TCTCAAAGAAACACGTGCCGC-3' and reverse primer SR: 5'-TCTCAAAGATATCGTTGCCGC-3' using Pyrobest DNA polymerase (TAKARA Bio Inc., Japan). The PCR product was extended with an A-tail by *Taq* DNA polymerase (Biostar, China), then cloned into a pGEM-T easy vector (Promega) and sequenced.

For the construction of a recombinant bacmid, an *hsp70-egfp-SV40* cassette was digested from the pKS-*egfp-cm^r* vector (kindly provided by Prof. Just M. Vlak, Wageningen University) by *Xho*I and *Hind*III, blunted by Klenow Fragment (Takara) and inserted into the *Bst*1107I site of the transfer vector pFast-BacTMDual (Invitrogen), to give pFastBacDual-*egfp*. Then, the CCHFV S gene was digested from pGEM-T Easy vector by *Eco*RI and subcloned into pFast-BacDual-*egfp*, generating donor plasmid pFastBacDual-*egfp-cchfvs*. The donor plasmid pFastBacDual-*egfp-cchfvs* was introduced into AcBacmid via Tn7-mediated transposition (Bac-to-Bac, Invitrogen), resulting in recombinant bacmid *Ac-egfp-cchfvs* (Fig.1). The recombinant bacmid was identified by PCR with M13 primers.

Virus propagation and titration

One µg of *Ac-egfp-cchfvs* DNA was transfected into 1×10⁶ Sf9 cells using 12 µL lipofectin reagent (Invitrogen), according to the Bac-to-Bac Expression Systems manual (Invitrogen). At 120 h post transfection (p.t.), 1 mL supernatants were centrifuged

at 3,000×g for 5 min to remove cell debris, and then infected in a 75 cm² cell culture flask (Corning) of Sf9 cells. Recombinant BVs were harvested at 168 h post infection (p.i.) and stored at 4 °C for usage. The titer of the recombinant virus was determined by an end-point dilution assay [15].

Electron microscopy of *Ac-egfp-cchfv* infected Sf9 cells

Sf9 cells were infected with *Ac-egfp-cchfv*s or control virus *Ac-egfp* at a MOI of 1 and 5, respectively. The cells were collected at 72 h p.i. and fixed for 16 h at 4 °C with 2.5% (W/V) glutaraldehyde in 0.1 mol/L PBS (pH7.2). The EM specimens were prepared according to Wang *et al.* (2010) [28] and recorded by transmission electron microscope (FEI Tecnai G2, operated at 200 kV).

Purification of Virus-Like Particles by sucrose-gradient ultracentrifugation

Eight flasks (1×10⁷ per each) of Sf9 cells were infected with *Ac-egfp-cchfv*s or *Ac-egfp* at a MOI of 1. The cells were collected at 72 h p.t. and broken by ultrasonic wave. The ultrasonic supernatants were layered on top of a 25% sucrose cushion, and spun for 2 h at 25,000 × g. The pellets were dissolved in 0.1× TE buffer (pH 7.2) and further purified by a sedimentation trough with 25% to 60% sucrose gradients (28,000 ×g, 3 h). Each band was collected separately.

Western blot analyses

The expression of the N protein in the infected Sf9 cells was examined by Western blot. Sf9 cells were infected with *Ac-egfp* or *Ac-egfp-cchfv*s at a MOI of 5 and cells were harvested at 72 h.p.i. The cell samples were disrupted in 4×SDS-PAGE sample buffer, and electrophoresed in 10% SDS-polyacrylamide gels. The proteins were transferred onto Hybond-N membranes

(Amersham) by semi-dry electrophoresis. Western blot was also used to detect N proteins in different fractions after ultracentrifugation purification. Western blotting was performed according to Wei *et al.* [31] with a slight modification. The primary antibody, anti-CCHFV N polyclonal rabbit serum [31] was used at 1:1000 dilution, and the secondary antibody, goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase was used at 1:2000 dilution. Finally, the signals were detected by NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, SABC, China). Two control antibodies, anti-GP64 antibody (rabbit antiserum against AcMNPV major envelope protein, 1:2000 dilution) [27] and anti-VP39 antibody (rabbit antiserum against AcMNPV nucleocapsid protein, 1:5000 dilution) [4] were used as controls to detect AcMNPV virions in ultracentrifugation fractions.

Negative stain and Immunogold-labeled EM (IEM) of VLPs

For negative staining, each band from the sucrose-gradient ultracentrifugation was collected and samples were adsorbed onto carbon-coated copper 200 mesh grids for 2 h at room temperature (RT). Grids were

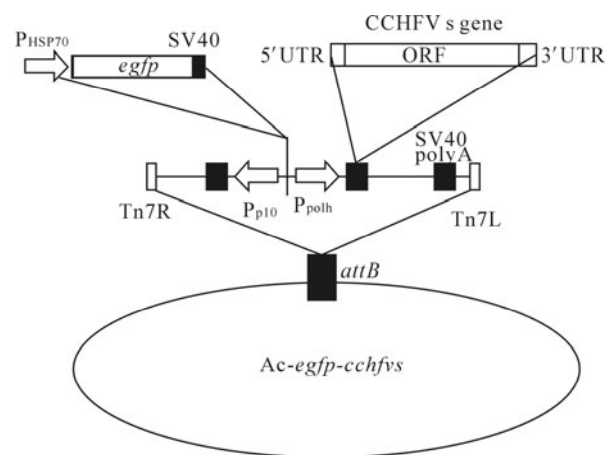


Fig. 1. The schematic showing construction of recombinant virus *Ac-egfp-cchfv*s.

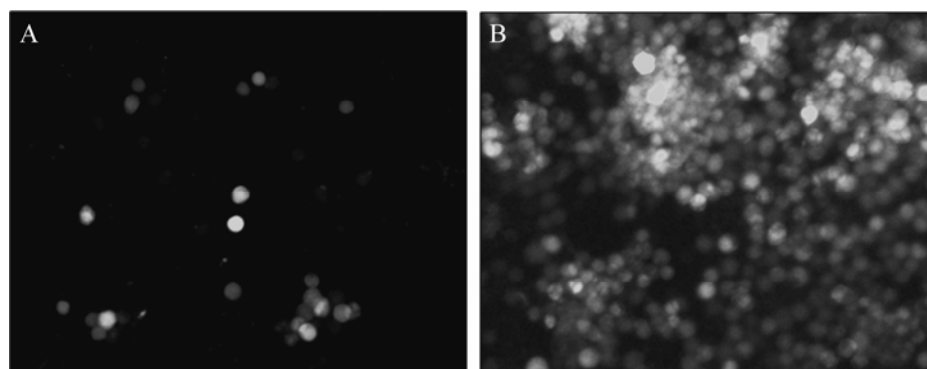


Fig. 2. Transfection of the recombinant bacmid. Sf9 cells were transfected with *Ac-egfp-cchfvs* bacmid DNA. The EGFP expression was observed at 72 h p. t. (A) and 100 h p. t. (B).

transferred into a drop of negative stain (2% phosphomolybdic acid, pH6.5) for 1 min.

IEM was performed according to Deng *et al.* [4]. Briefly, Nickel grids were floated for 2 h in a 20 μ L drop of sample to adsorb the particles, and then the Grids were incubated with anti-CCHFV N polyclonal antibody (1:20 diluted in 5% [W/V] bovine serum albumin-Tris-Buffered-Saline [BSA-TBS]) for 2 h at room temperature (RT) in a wet chamber. Preimmune sera were used as negative controls. Grids were washed six times with $0.1 \times$ TE buffer and then incubated for 2 h with 12-nm Colloidal Gold-AffiniPure goat anti rabbit immunoglobulin G (Jackson Immuno Research) diluted 1:50 at RT. Grids were then washed with $0.1 \times$ TE buffer and stained as described above. Specimens were observed under a transmission electron microscope (FEI Tecnai G2) operated at 200 kV.

RESULTS

Generation of recombinant AcMNPV containing CCHFV *s* gene.

The recombinant bacmid *Ac-egfp-cchfvs* were generated as described in Materials and Methods and its genomic structure is shown in Fig. 1A. Positive clones were confirmed by PCR amplifications and

sequencing (data not shown). Sf9 cells were transfected with the recombinant bacmid DNA. At 72 h p.t., green fluorescence was found in some cells (Fig. 2A), suggesting the successful transfection of recombinant bacmid DNA into the Sf9 cells. At 100 h p.t., fluorescence spread from cell to cell (Fig. 2B), indicating the propagation of infectious progeny recombinant *Ac-egfp-cchfvs*.

The expression of CCHFV nucleocapsid protein in insect Sf9 cells.

To detect the expression of N proteins in Sf9 cells, Western blot analysis was conducted. As Fig. 3 shows, anti-CCHFV N antibody can detect a band corresponding to the predicted molecular mass (~54 kDa) of N protein in *AcBac-egfp-cchfvs* infected cells (Fig. 3, lane 3), but not in the control virus *Ac-egfp* infected Sf9 cells (Fig. 3, lane 2) or Sf9 cells control (Fig. 3, lane 1). Thus, Western analysis confirmed the correct

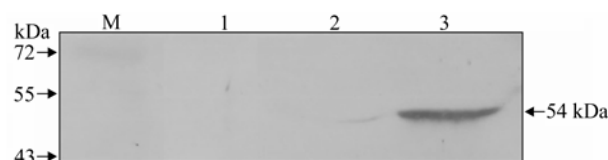


Fig. 3. Western blot analysis on the expression of CCHFV N protein in Sf9 cells. Lane 1, Sf9 cells control; lane 2, Sf9 cells infected with *Ac-egfp*; lane 3, Sf9 cells infected with *Ac-egfp-cchfvs*. M, pre-stained protein marker.

expression of CCHFV N protein by the baculovirus-insect expression system.

CCHFV nucleocapsid proteins self-assemble into Virus-Like Particles (VLPs) in insect cells.

The nucleocapsid protein of some viruses, *i.e.* Dengue virus-2, Hepatitis C virus (HCV), can automatically assemble into Virus-like particles [16, 20]. To detect whether CCHFV N proteins can self-assemble into VLPs, Sf9 cells infected with *Ac-egfp-cchfvs* at different MOIs (1 or 5) were observed by transmission electron microscopy. A cluster of sphere-like particles, which appear like VLPs were found in vesicles (Fig. 4A and B, boxed region) in the cytoplasm of Sf9 cells both infected at MOI of 1 and 5. The numbers and density of sphere-like particles forms in Sf9 cells infected with a MOI of 5 seems to be higher than

those infected at a MOI of 1 (Fig. 4 A' and B'). These particles varied both in morphology and size, ranging from 40 nm to 160 nm in diameter. In contrast, there were very few sphere-like particles present in the cytoplasmic vesicles in control virus *Ac-egfp* infected Sf9 cells, at an MOI of either 1 or 5 (data not shown).

To further confirm whether the sphere-like particles were composed of CCHFV N proteins, *Ac-egfp-cchfvs* or *Ac-egfp* infected cell lysate was analyzed by sucrose-gradient ultracentrifugation, respectively. Three major bands, upper (~30% sucrose density), middle (between 35%-45%) and lower (between 50%-55%) were visible in *Ac-egfp-cchfvs* infected cell sample after centrifugation (Fig. 5A). However, only one band (~45%) was detected in the *Ac-egfp* infected cell sample, and EM analysis confirmed this

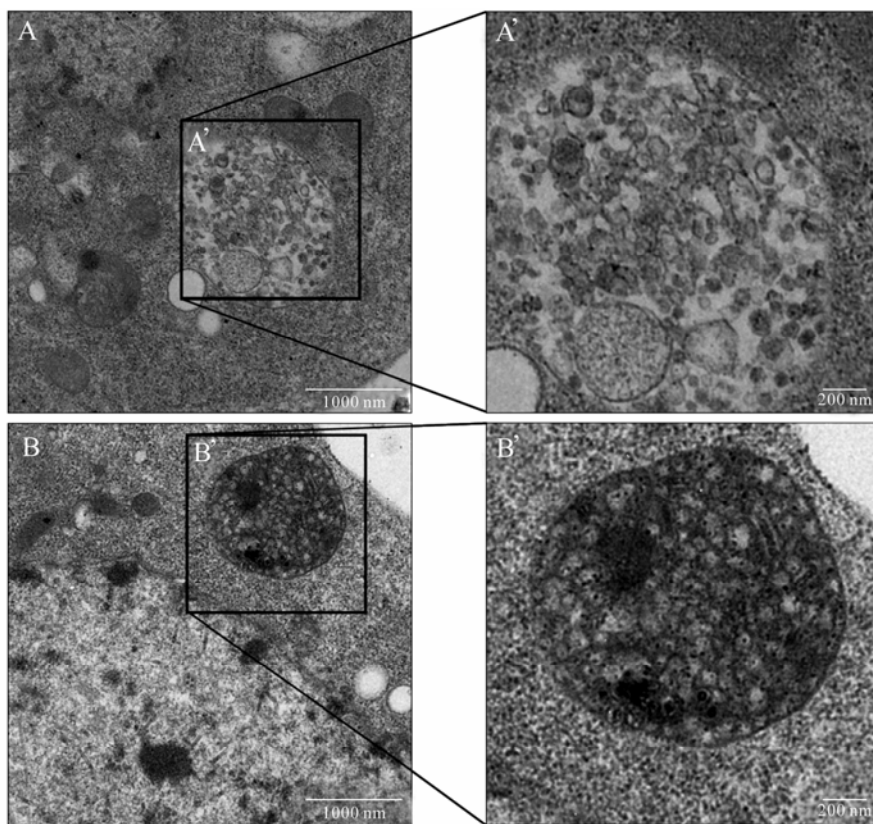


Fig. 4. Electron microscopy of Sf9 cells infected with *Ac-egfp-cchfvs* at a MOI of 1 (A) and 5 (B). A' and B' show an enlarged view of the boxed region of A and B, respectively.

band was composed mostly of rod-shape AcMNPV, not sphere-like particles (data not shown), and thus it was not subjected to further analyses. This result is in agreement with the above EM result that very few sphere-like particles were detected in Sf9 cells infected by *Ac-egfp*.

Each centrifugation band in *Ac-egfp-cchfvs* infected cell samples was collected for Western and EM analysis. Western blot demonstrated the presence of CCHFV N protein in all three purified bands with most the abundance in the upper band (Fig.5B, lane 3-5). It was also found in the positive control virus *Ac-egfp-cchfvs* infected cells (Fig. 5B, lane 2), but not in the negative control virus *Ac-egfp* infected cells (Fig. 5B, lane 1). Antibodies against AcMNPV budded virus (BVs) envelope fusion protein GP64 and viral nucleocapsid protein VP39 were also used for Western blot to detect baculoviruses in those samples

(Fig. 5C). Both GP64 and VP39 were detected in the upper and middle bands (Fig. 5C, lane 3 and 4), suggesting the presence of AcMNPV BVs in upper and middle centrifugation fractions; VP39 but not GP64 was detected in lower band as well (Fig. 5C, lane 5), indicating the existence of AcMNPV occlusion derived virions (ODVs) in this band.

Negative staining results demonstrated the upper band was mainly composed of sphere-like particles, with small amounts of AcMNPV BVs (Fig. 6A), and the lower band was mainly constituted of AcMNPV ODVs (data not shown). The EM results were in consistent with those of Western blot analyses. The upper band was further analyzed by IEM. As Fig. 6D showed, gold probes specifically surrounded the sphere-like particles when using anti-CCHFV N antiserum as the primary antibody. The pre-immune rabbit serum and no primary antibody were used as

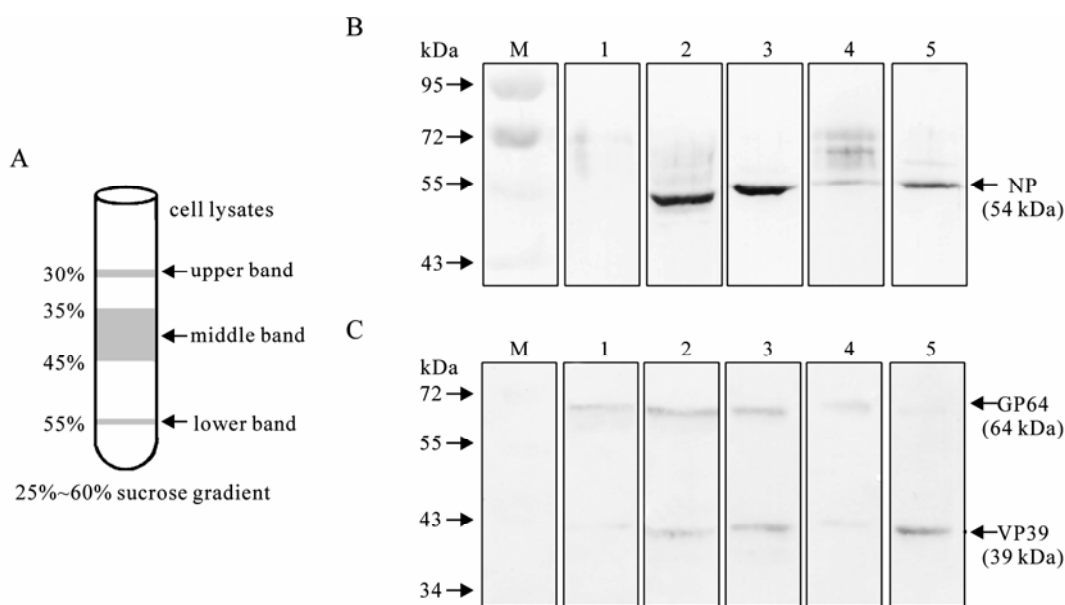


Fig. 5. Western blot analyses of gradient-ultracentrifugation fractions. A: Schematic showing sucrose-gradient ultracentrifugation fractions of Sf9 cells infected with *Ac-egfp-cchfvs*. B and C: Western blot results of each ultracentrifugation fraction probed with anti-CCHFV N antibody (B) or anti-AcMNPV GP64 and VP39 antibodies (C). Lane 1, Sf9 cells infected with *Ac-egfp*; lane 2, Sf9 cells infected with *Ac-egfp-cchfvs*; lane 3, the upper band of cell lysate; lane 4, the middle band of cell lysate; Lane 5, the lower band of the cell lysate; M: pre-stained protein maker.

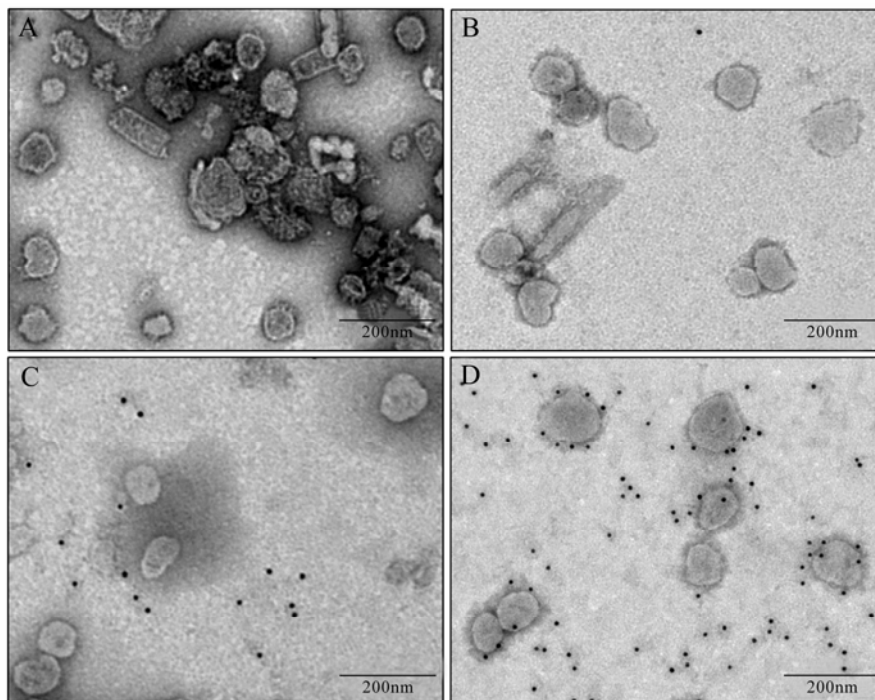


Fig. 6. EM and IEM analyses of CCHFV VLPs (upper band from ultracentrifugation). A: Negative staining electron micrograph of CCHFV VLPs. B, C and D: IEM micrographs of CCHFV VLPs probed with no primary antibody (B), pre-immunized rabbit serum (C) and anti-CCHFV N antibody (D) as the primary antibody.

negative controls (Fig. 6B and C). These results confirmed the sphere-like particles formed in the *Ac-egfp-cchfvs* infected cells were made up of N protein. Thus, expressing CCHFV N protein by baculovirus can form VLPs in insect cells.

DISCUSSION

In this paper, we present evidence that VLPs are formed when Sf9 cells are infected with a recombinant baculovirus expressing CCHFV N protein. After sucrose-gradient ultracentrifugation the VLPs were further confirmed by Western blot and IEM.

As a major component of CCHFV virion, the nucleocapsid protein plays important roles during virus assembly and morphogenesis^[32]. The N proteins bind with viral RNAs and interact with RdRp (RNA dependent RNA polymerase, also called L protein) to form RNP complexes^[1,10], then the RNPs are

packaged with bilayer lipid membrane embedded with glycoproteins^[24]. Our results indicated that N proteins may self-assemble into nucleocapsids in natural CCHFV infection. However, the specific mechanism of CCHFV assembly remains to be investigated.

Many viral nucleocapsid proteins or envelope glycoproteins can spontaneously self-assemble into VLP. VLP has been developed as an ideal vaccine candidate, because it mimics the overall structures of a virus particle without containing infectious genetic materials, thus is safe and highly immunogenic. Among a variety of protein expression systems for VLP production, the baculovirus-insect system has many advantages, being safe, cheap and easy to scale-up. So far, more than 30 VLPs among 15 virus families have been produced by this system and demonstrated to be highly immunogenic and potential vaccine candidates, including members of *bun-*

yaviridae family, Rift Valley fever virus (RVFV) and Hantaan virus [18]. Recently, the highly conserved region 235 to 305 aa of CCHFV (YL04057 strain) N protein was identified as a highly antigenic region [31]. Therefore, the VLP generated in this research may provide a promising vaccine candidate for CCHF prevention. Further experiments will be conducted to test the immunogenicity of CCHFV VLPs in animal models.

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