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Formation of peste des petits ruminants spikeless virus-like particles by co-expression of M and N proteins in insect cells

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ABSTRACT

Peste des petits ruminants virus (PPRV) has a non-segmented negative sense RNA genome and is classified within the *Morbillivirus* genus of the *Paramyxoviridae*. Using the Bac-to-Bac® baculovirus expression system, we constructed recombinant baculoviruses that were able to co-express the PPRV matrix and nucleocapsid proteins in insect cells under the control of the polyhedron and p10 promoters, respectively. The results showed that although both structural proteins were expressed at a relatively low level, the interaction between them caused the formation of virus-like particles (VLPs) by viewing of transmission electron microscopy. The VLPs morphologically resembled authentic PPRVs but lacked spikes protruding from the particulate surfaces. Interestingly, the diameter of PPRV VLPs ranged from 100 to 150 nm, far less than the mean diameter (400–500 nm) of parental virions.

Peste des petits ruminants (PPR) is a highly contagious disease responsible for high morbidity and mortality in sheep and goats (Abubakar et al., 2009). This disease is caused by peste des petits ruminants virus (PPRV), which is classified into the genus *Morbillivirus* in the family *Paramyxoviridae*, as its biological and physicochemical features are closely related to the other morbilliviruses (Gibbs et al., 1979). The full genome of PPRV, consisting of a single-stranded negative sense RNA, encodes six structural proteins: the nucleocapsid protein (N), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the haemagglutinin protein (H) and the polymerase protein (L) (Banyard et al., 2010). Structurally, PPRVs are morphologically pleomorphic particles (400–500 nm) enveloped by lipid membrane with viral glycoproteins, seen as peplomers protruding from the envelope. The M protein lies beneath the virion envelope and interacts with the internal nucleocapsid and the cytoplasmic tails of the surface glycoproteins (Mahapatra et al., 2006).

To date, baculovirus expression system has become one of the most versatile and robust eukaryotic expression systems for foreign protein expression, including the interferon (Chen et al., 2011), antigen (Yousefi et al., 2012), enzyme (Brading et al., 2012) and so forth. This system permits proper post-translational modifications in insect cells, such as protein folding (Ailor and Betenbaugh, 1999), proteolytic processing (O'Reilly et al., 1994), glycosylation (Lopez et al., 1999; Jarvis, 2003; Tomiya et al., 2003) and disulfide bond formation (Choi et al., 2004; Kushima et al., 2010), many of which may be identical to those occurring in mammalian cells. Efficient expression of foreign proteins and proper post-translational modifications in insect cells contribute to the formation of virus-like particles (VLPs) (Liu et al., 2013b). The VLPs generally consist of viral proteins that self-assemble into replication-incompetent particulates closely resembling the authentic virions. In this study, using the Bac-to-Bac® baculovirus expression system (Life technologies), we produced PPRV China/tibet/geg/07–30 strain (GenBank Access No. FJ905304.1) VLPs, which contained the PPRV M and N proteins. Construction and identification of the VLPs were performed as follows.

The open reading frames (ORFs) of M and N genes were cloned into the *Bam*H I/*Spe*I and *Nhe*I/*Kpn*I restriction sites in pFastBac™ Dual, respectively. The positive clones (Dual-MN) were confirmed by polymerase chain reaction (PCR) amplification, followed by sequencing. The positive Dual-MN was used for generating a recombinant bacmid by site-specific transposition in *Escherichia coli* DH10Bac cells (Luckow et al., 1993; Mallick et al., 2011). The positive transformants were analyzed by PCR using the pUC/M13 forward primer: 5′-CCCAGTCACGACGTTGTAAAACG-3′ and reverse primer: 5′-AGCGGATAACAATTTCACACAGG-3′. The PCR yielded a 5.1 kb amplicon (Fig. 1A) corresponding to the sequence of interest, followed by sequencing.

Positive recombinant bacmids were purified and then transfected into *Spodoptera frugiperda* (Sf9) cells by the Cellfectin II reagent

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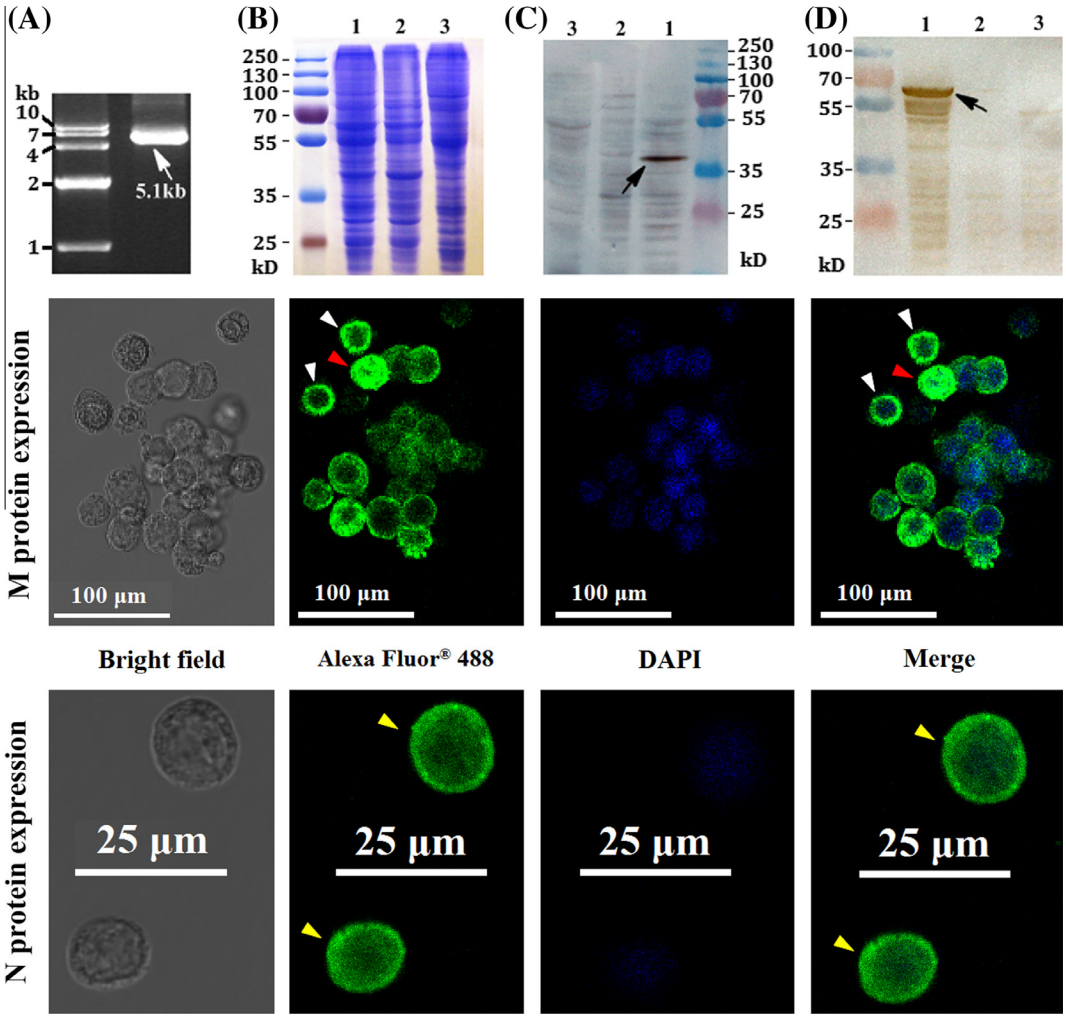

Fig. 1. Generation and characterization of recombinant baculovirus co-expressing the M and N proteins in Sf9 cells. PCR analysis of recombinant bacmid comprising the M and N ORFs was performed using the pUC/M13 forward/reverse primers, showing a 5.1 kb positive band indicated by a white arrow (A). The M and N proteins were expressed at a relatively low level in Sf9 cells, so that no corresponding bands were detectable in the polyacrylamide gel by staining with Coomassie brilliant blue (B). However, a 38 kD (C, arrowed) and a 58 kD (D, arrowed) bands corresponding to the M and N proteins were observed on the transfer membranes by Western blot, respectively. Lane 1 and 2, Sf9 cells 72 h post-infection with recombinant and wild-type baculoviruses, respectively. Lane 3, uninfected Sf9 cells. Immunofluorescent analyses of the M (middle panel) and N (lower panel) proteins expressed in Sf9 cells were performed by confocal microscopy using the anti-M polyclonal and anti-N monoclonal antibodies as primary antibodies, and Alexa Fluor® 488-conjugated antibodies as secondary antibodies, respectively. Nuclei were stained with DAPI. Images of staining with Alexa Fluor® 488 and DAPI were merged using Adobe Photoshop software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Life technologies). The bacmid-transfected Sf9 cells were maintained for 72 h followed by cytopathic effects, indicating that budded first-generation (P1) recombinant baculoviral stock would be released into the medium. The P1 viral stock was obtained and added to a healthy Sf9 cells monolayer and maintained for at least 72 h to generate P2 viral stock. The resulting P2 viral stock was used to infect a 100 mL suspension culture of Sf9 cells to amplify the P2 to P3 viral stock. The P3 viral stock was titrated by plaque assay on Sf9 cells cultured in a 6-well plate. The titer of P3 viral stock reached appropriately 1×10^8 PFU/mL.

Sf9 cells were cultured to 70% confluence in each well of a 6-well plate. The P3 viral stock solution was added to four wells; wild-type baculovirus-infected and uninfected Sf9 cells were used as controls in the other two wells. Cells were cultured at 27 °C and collected 72 h post-infection, followed by washing twice with PBS and centrifugation at 1000 rpm for 5 min at 4 °C. Pellet of cells was resuspended in pH 7.4 phosphate buffered saline (PBS) and mixed with loading buffer (Takara) heated at 95 °C for 10 min, followed by analysis of SDS–PAGE. Subsequently, the gels were used for staining with Coomassie brilliant blue and for analysis of Western blot as described previously (Liu et al., 2013a), respectively. Anti-M polyclonal (Liu et al., 2013a) and anti-N monoclonal antibodies were used as primary antibodies, and anti-mouse IgG-peroxidase (Sigma) served as the secondary antibody to examine the expression of the M and N proteins in Sf9 cells, respectively. The results showed that although expressions of both proteins were too relatively low to visualize two bands in a polyacrylamide gel (Fig. 1B), a 38 kD (Fig. 1C, arrowed) and a 58 kD (Fig. 1D, arrowed) bands corresponding to the M and N proteins respectively, were observed on transfer membranes by the Western blot.

In order to detect the expression and localization of both proteins in Sf9 cells, immunofluorescence assay was carried out as described previously (Calvert et al., 2012), using the anti-M polyclonal and anti-N monoclonal antibodies as primary antibodies, and Alexa Fluor® 488 donkey anti-mouse antibodies (Life technologies) as secondary antibodies. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) (Sigma). Image acquisition was performed with a confocal microscope (Leica TCS SP5 II). The wild-type baculovirus-infected Sf9 cells were analyzed in parallel using a combination of anti-M polyclonal and anti-N monoclonal