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#### **BRIEF REPORT**



## Generation and immunogenicity of virus-like particles based on mink enteritis virus capsid protein VP2 expressed in Sf9 cells

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#### Abstract

Mink enteritis virus (MEV) is a parvovirus that causes acute enteritis in mink. The capsid protein VP2 of MEV is a major immunogenicity that is important for disease prevention. In this study, this protein was expressed in *Spodoptera frugiperda* 9 cells using a recombinant baculovirus system and was observed to self-assemble into virus-like particles (VLPs) with a high hemagglutination (HA) titer (1:2<sup>16</sup>). A single-dose injection of VLPs (HA titer, 1:256) resulted in complete protection of mink against virulent MEV challenge for at least 180 days. These data suggest that these MEV VLPs could be used as a vaccine for the prevention of viral enteritis in mink.

Mink enteritis virus (MEV) is a non-enveloped virus belonging to the genus *Protoparvovirus*. MEV possesses a linear single-stranded negative-sense DNA genome of approximately 5 kb [1]. The MEV genome encodes two nonstructural proteins (NS1 and NS2) and two capsid proteins (VP1 and VP2) [1–3]. The parvovirus capsid is composed of approximately 60 copies of VP2 and 10 copies of VP1 [4–6]. The VP2 capsid protein is highly antigenic [7, 8] and has important roles in determining the host range [9] and tissue tropism of the virus [3, 10, 11]. Some regions of the VP2 capsid protein serve as targets for neutralizing antibodies [12, 13].

Mink viral enteritis (MVE) caused by MEV is an acute highly contagious enteric disease that was first reported in Canada [11, 14]. It is one of the most important viral diseases of mink and has a worldwide distribution [1, 15],

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<sup>2</sup> Changchun SR Biological Technology Co., LTD, Changchun 130012, Jilin, China causing serious economic loss within the industry. The main clinical signs of MVE are vomiting, severe diarrhea, anorexia and depression [16]. MEV can infect mink of all ages; however, newborn and juvenile animals are the most susceptible [17]. Due to the severity of the disease and the potential economic impact, prevention is preferred to treatment. Currently, inactivated or modified live MEV vaccines are widely used in farmed animals to control MEV infection. The drawbacks with these vaccines are the potential risk of reversion to virulence in the case of the modified live vaccines and the risk of incomplete inactivation and loss of immunogenicity during the inactivation process in the case of the killed vaccines [18].

A major safety feature of the insect baculovirus is that it does not replicate in mammalian cells [19]. Furthermore, the baculovirus expression vector system has the advantage that the expressed proteins are posttranslationally modified in a similar way to proteins expressed in mammalian cells [20]. These features have made the baculovirus expression vector system a popular choice for the production of virus-like particles (VLPs) [19, 21–23].

VLPs, which are non-infectious due to the lack of a viral genome, are self-assembled structures of one or more viral structural proteins that simulate the organization of native viruses. VLPs can also offer advantages over conventional inactivated or subunit vaccines in immunogenicity by inducing both cellular and humoral immune responses [18, 24–27]. They have been used as the basis of vaccines for human use, including the human papilloma virus vaccine

Cervarix<sup>TM</sup> (GlaxoSmithKline) [19]. In this study, a recombinant baculovirus expressing MEV VP2 was constructed, MEV VLPs were produced in *Spodoptera frugiperda* 9 (Sf9) cells using a baculovirus expression system, and the immunogenicity of the MEV VLPs vaccine in mink was investigated.

The virulent MEV strain JL (GenBank accession no. MT250783) was isolated from a clinical sample in Jilin Province, China, using a method reported previously [28]. The codon usage of the VP2 gene of MEV strain JL was optimized to match the codon bias of Sf9 cells. To generate the recombinant transfer plasmid pFastBacDual-dVP2, two copies of the optimized VP2 gene were cloned into the

*XhoI/Nhe*I sites and *NotI/Hind*III sites, respectively, of the baculovirus transfer vector pFastBac<sup>TM</sup> Dual. Then, pFastBacDual-dVP2 was introduced by transformation into *E. coli* DH10 Bac/AcMNPV competent cells for VP2 gene transposition into the bacmid to generate bacmid-dVP2 (Fig. 1A). To generate the recombinant baculovirus (bacMEV-VP2), Sf9 cells were transfected with 4  $\mu$ g of bacmid-dVP2 using Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. The supernatant containing recombinant baculovirus was harvested at 5-6 days post-transfection, resulting in an obvious cytopathic effect (CPE), which included an increase in cell diameter, the appearance of vesicles (Fig. 1B), and detachment from



**Fig. 1** Construction and identification of MEV VLPs. (A) Schematic representation of the procedure for VLP construction. (B) Identification of the recombinant baculovirus bacMEV-VP2 under a light microscope. Left, Sf9 cells infected with bacMEV-VP2 for 96 h; right, control Sf9 cells with no baculovirus infection. The scale bar indicates 50 nm. (C) Identification of MEV-VP2 protein expressed in Sf9 cells using an indirect immunofluorescence assay. Left, Sf9 cells infected with bacMEV-VP2; right, control Sf9 cells with no baculovirus infection. The scale bar baculovirus infection. The cells were stained with Evans blue. The scale bar indicates 50 nm. (D) Identification of MEV-VP2 protein

by SDS-PAGE (left) and western blot (right). The molecular mass of VP2, indicated by an arrow, is 65 kDa. Lane M, protein molecular weight marker. Lanes 1 and 4, Supernatant from NaHCO<sub>3</sub>-treated mock-infected Sf9 cells. Lanes 2 and 3, the supernatant from NaHCO<sub>3</sub>-treated Sf9 cells infected with bacMEV-VP2. Lane 5, F81 cell lysate after infection with wild-type MEV. (E) Morphology of VLPs. Left, wild-type MEV; middle, VLPs in the supernatant of a lysate of Sf9 cells infected with bacMEV-VP2; right, immunoelectron microscopy of VLPs the plate. The titer of the recombinant baculovirus was determined using a Baculovirus Rapid Titer Kit (TaKaRa, Japan) according to the instructions. The recombinant baculovirus bacMEV-VP2 was used to infect Sf9 cells for the further identification.

The expression of the MEV VP2 capsid protein in Sf9 cells was confirmed by indirect immunofluorescence assay (IFA) at 48 h after infection with the recombinant baculovirus. Specific green fluorescence could be observed in Sf9 cells infected with bacMEV-VP2, but not in control cells (Fig. 1C). For further identification of protein expression, Sf9 cells in suspension were infected with bacMEV-VP2 at a multiplicity of infection of 0.1 and observed daily. The infected cells, which exhibited an increase in volume, were harvested at 96 h postinfection. After centrifugation at 2,935  $\times$  g for 10 min, the cells were lysed by resuspending them in 0.025 M NaHCO<sub>3</sub> and placing them on ice for 30 min. The supernatant of the NaHCO<sub>3</sub>-treated infected cells was then collected and analyzed by SDS-PAGE (Fig. 1D, lanes 1 and 2), in which the target band (molecular weight of about 65 kDa) was clearly visible. The target band of VP2 was further identified by western blot (Fig. 1D, lanes 3 and 4) using a guinea pig anti-MEV serum (1:500) as a primary antibody and horseradish peroxidase (HRP)-labeled goat anti-guineapig IgG (Invitrogen, USA) as a secondary antibody. The size of the target band was similar to that of the wild-type MEV (Fig. 1D, lane 5) which was stained using Electrochemiluminescence (ECL) Western Blotting Substrate (Pierce, Rockford, USA), and its image was captured using a Tanon-5200 Chemiluminescent Imaging System (Tanon Science & Technology Co., Ltd). These results indicated that the VP2 protein of MEV was successfully expressed from the recombinant baculovirus bacMEV-VP2.

To investigate whether capsid-like particles were selfassembled correctly, the supernatant of NaHCO<sub>3</sub>-treated infected cells was analyzed by electron microscopy. The result showed that the VP2 capsid protein expressed in Sf9 cells formed VLPs with a diameter of about 20 nm (Fig. 1E) whose morphology was similar to the wild-type MEV virus (Fig. 1E). Interestingly, VLPs were easily found in the supernatant obtained after cell lysis by electron microscopy, without VP2 protein separation or manual intervention. Furthermore, VLPs were identified by immunoelectron microscopy (IEM, USA) using guinea pig anti-MEV serum as a primary antibody and a gold-tagged anti-guinea pig antibody as a secondary antibody. There results showed that the VLPs were able to bind with the MEV-specific antibody (Fig. 1E). For further characterization, a hemagglutination (HA) test was performed at 4 °C using a 1% (v/v in PBS) suspension of pig erythrocytes as described previously [29]. The HA titer of the MEV VLPs was found to be as high as  $1:2^{16}$ , which is higher than that observed in cultivated mammalian cells  $(1:2^{10})$ . These results demonstrate that MEV

VP2 expressed by recombinant baculovirus can self-assemble into VLPs with physical and immunological properties similar to those of the native virus.

To test the immunogenicity of the MEV VLPs, a vaccine formulation was prepared containing MEV VLPs (HA titer adjusted to 1:256) and aluminium hydroxide gel. Twentyfive 50-day-old minks that were seronegative for MEV were randomly divided into five groups. Groups 1 and 2 (five minks in each group) were inoculated intramuscularly with a single dose of MEV VLP vaccine (1.0 ml) per animal. Groups 3 and 4 (five minks in each group) were injected with PBS as a negative control. Blood samples were taken from groups 1 and 3 at 0, 7, 14, 30, 60, 90, 120, 150, 180 days post-vaccination (dpv). Serum antibody titers against MEV were determined by hemagglutination inhibition (HI) assay as previously described [29]. Briefly, serial two-fold dilutions of inactivated serum samples were made with PBS and mixed with 8 HA units of MEV. After incubation at 37°C for 30 min, 1% pig erythrocytes were added, and the samples were incubated at 4°C for another 90 min. The titer was calculated as the highest dilution at which agglutination was still observed. This assay showed that an antibody response was elicited at 7 days post-immunization, with a titer above 1:32. The HI titer reached its maximum value (1:1024) within 30 days. Moreover, the antibody HI titer was maintained at no less than 1:32 for at least 180 days (Fig. 2A).

The safety of the vaccine was tested in group 5 (5 minks) by inoculation with an overdose of MEV VLPs (2.0 ml). The animals' demeanor, intake of food and fluid, and excretion were observed daily. The animals in group 5 were euthanized at 14 days post inoculation and dissected to look for any abnormal occurrences at the injection sites. In addition, a muscle section from the injection site of each animal was examined in a histopathological assay using hematoxylineosin (HE) staining. The intestinal tissues were examined using both HE staining and immunohistochemistry (IHC) assay. Continuous observation of the animals' behavior for 14 days did not reveal any abnormal clinical signs (parameters included depression, food and fluid intake and excretion) Furthermore, the histopathological examination did not reveal any abnormalities, either generally or at the injection site (Fig. 2B). No abnormalities were observed in muscle sections taken from the injection site (Fig. 2B) or in intestinal tissues (Fig. 2C). These data showed that the vaccine is safe in mink.

To determine the protection provided by the MEV VLPs vaccine, minks in groups 2 and 4 were challenged orally with  $1.5 \times 10^{7.5}$  **22** times the median tissue culture infectious dose**22** (TCID<sub>50</sub>) of the virulent MEV strain JL at 14 dpv, and the minks in groups 1 and 3 were challenged with the same dose of MEV strain JL at 180 dpv. No adverse clinical signs (depression, appetite, diarrhea and death)

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B Safety evaluation at the injection site



C Safety evaluation at intestinal tissues



**Fig. 2** Immunogenicity and safety of VLPs. (A) HI titer determination. The HI titer in the serum of minks in groups 1 and 3 was measured using pig erythrocytes. (B) Safety evaluation at the injection site. Minks in group 5 were dissected at 14 dpv to look for abnormalities at the injection site (left), and a muscle section from the injection site was analyzed by HE assay (right). (C) Safety evaluation using intestinal tissues. Minks in group 5 were dissected at 14 dpv, and intestinal tissues were analyzed by HE (left) and IHC (right) assay

were observed in any of the vaccinated minks. In contrast, all of the minks in the control group showed typical symptoms of MEV, such as depression, anorexia and diarrhoea. At 10 days post-challenge, the mortality rates in groups 4 (14 dpv) and 3 (180 dpv) were 60% and 20%, respectively (Fig. 3A). In order to test whether the vaccine was able to reduce the amount of virus shedding, we measured MEV antigen excretion in fecal samples at 0-7 days after challenge using the HA test and quantitative PCR (qPCR). The

sequences of the primers and probe were as follows: FQ-MEV-F. 5'-CAGGAATTAACTATACTAATATAT TTAATA -3'; FQ-MEV-R, 5'-AAATTTGACCATTTGGATAAACT-3'; MEV-probe, FAM-TGGTCCTTTAACTGCATTAAA TAATGTACC-BQ1. The results showed that the virus could be detected in the control group for at least 6-7 days after challenge, whereas the vaccinated minks had low fecal HA titers ( $\leq 1$ :16) (Fig. 3B). The trend of qPCR was consistent with that of HA, but with higher sensitivity. The amount of viral genome detected by qPCR was much lower than that of control group (Fig. 3C). These results indicate that the vaccine can effectively reduce the virus titer and the time of viral shedding. Reducing viral shedding is important for prevention of MEV transmission by the fecal-oral route [30]. When the intestinal tissues in vaccinated (MEV VLPs) and unvaccinated (control) groups were examined by HE stains and IHC assay, no abnormalities were observed in MEV VLPs group, but However, in the control group, the epithelial cells and lamina propria cells in the upper segment of villi were exfoliated and necrotic in both HE and IHC staining (Fig. 3D). The above results indicated that a single dose of MEV VLP vaccine could induce a high HI titer against MEV and that the immune response was sufficient to protect minks from challenge for at least 180 days.

The use of a baculovirus expression system to produce MEV VLPs was first described by Christensen et al. [31], who showed that subcutaneous immunization with 40,000 HA units of VLPs could induce a measurable antibody response and confer resistance to challenge with virulent MEV. However, the yield of the VLPs was not mentioned. Dalsgaard et al. successfully expressed an MEV VP2 epitope on the surface of recombinant cowpea mosaic virus propagated in black-eyed bean, (Vigna unguiculata) [30]. One subcutaneous injection of 1.0 mg of the chimeric virus particles protected minks from challenge with virulent MEV. Langeveld et al. developed a synthetic peptide vaccine and an epitope vaccine that protected minks against MEV infection [8]. In the present study, MEV VLPs were produced at a high yield (HA up to 1:2<sup>16</sup>). Moreover, the VP2 protein alone (in the absence of VP1) was able to assemble into VLPs with apparently normal morphology and good immunogenicity, which is consistent with previous reports describing the assembly of VLPs based on the VP2 proteins of other parvoviruses, such as canine parvovirus [29], porcine parvovirus [32], and goose parvovirus [33]. This property has been exploited for preparation of recombinant vaccines carrying foreign proteins [34]. The high yield of MEV VLPs is a distinct advantage, as it can reduce production costs significantly. Sf9 insect cells are ideal for largescale fermentation and can be used in commercial production systems.

Antibody detection (ELISA, HI and virus neutralization) is often used to assess the immunogenicity of a vaccine,



**Fig. 3** Survival and antigen excretion of immunized minks after challenge with virulent MEV. (A) Survival rate after challenge. (B) Antigen excretion in feces, detected using an HA test. (C) Antigen excre-

and passive transfer studies in dogs have shown that protection against canine parvovirus (CPV) is probably mediated through the humoral route [35, 36], and it is therefore likely that the protection against MEV is also mediated by the humoral route. The HI test is regarded as the gold standard for quantitative determination of antibody titers [37]. In the immunogenicity test of "Feline infectious enteritis (feline panleukopenia) vaccine (inactivated)" in European Pharmacopoeia, one of the recommended methods for measuring HI and virus-neutralizing antibodies was sufficient for vaccine evaluation. Therefore, in this study, the same HI test was used to determine the antibody titer.

Amino acids at different positions in the VP2 protein influence its structure and antigenicity [38, 39]. This can be helpful for understanding differences in the effectiveness of vaccines based on different strains [13]. Here, the MEV VLPs constructed based on the VP2 protein of MEV strain JL showed high antibody levels after immunization, and an antibody response could be detected within one week. After challenge with virulent MEV, the control



tion in feces, detected using qPCR. (D) Intestinal tissues analyzed by HE and IHC assay

group developed MEV symptoms within 7 days, with mortality rates between 20% and 60%, while all members of the vaccinated group survived and excreted only low levels of MEV in their feces, which is consistent with a previous report [31]. Moreover, the amount of VLPs with an HA titer of 1:256 was able to induce a high level of antibody (>1:80) for up to 150 days, and protection against challenge was sustained up for at least 180 days. Furthermore, there were no adverse reactions after vaccination. Future studies of this VLP-based vaccine will include an investigation of the cellular immune response it induces and a comparison of its immunogenicity with that of the current inactivated virus vaccine and modified-live vaccine.

In conclusion, the MEV VLP vaccine developed using a baculovirus expression system in Sf9 cells may be a safe and effective vaccine for preventing disease associated with MEV.

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#### **Compliance with ethical standards**

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

**Research involving animals** The animal experiments were approved by the Animal Care and Ethics Committee of the China National Research Center for Veterinary Medicine.

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