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Original article

Construction of a recombinant baculovirus expressing swine hepatitis E Virus ORF2 and preliminary research on its immune effect

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Abstract

In the swine hepatitis E virus (HEV), open reading frame 2 (ORF2) is rich in antigenic determinants and neutralizing epitopes that could induce immune protection. We chose the Bac-to-Bac[®] Baculovirus Expression System to express fragments containing the critical neutralizing antigenic sites within the HEV ORF2 protein of pigs to obtain a recombinant baculovirus. The fragment of swine HEV ORF2 region (1198-1881bp) was cloned into vector pFastBac[™]. A recombinant baculovirus, rBacmid-ORF2, was obtained after transposition and transfection. The molecular mass of the recombinant protein was 26 kDa. Mice were immunized by the intraperitoneal and oral routes with cell lysates of recombinant baculovirus rBacmid-ORF2. Serum and feces of the mice were collected separately at 0, 14, 28, and 42 d after immunization and the antibody levels of IgG and secretory IgA against swine HEV were determined using an enzyme-linked immunosorbent assay. The results suggested that rBacmid-ORF2 induced antibodies of the humoral and mucosal immune responses in mice and that the oral route was significantly superior to the intraperitoneal route. This is the first study to demonstrate that that recombinant baculovirus swine HEV ORF2 could induce humoral and mucosal immune responses in mice.

Key words: swine HEV, ORF2, baculovirus, immunogenicity

Introduction

Hepatitis E (HE) is a zoonotic disease caused by hepatitis E virus genus (HEV) genotypes (I-IV), which are considered as the same serotypes (Bautista et al. 1999). Humans and other mammals (especially pigs) are the primary sources of HEV infection (Vasickova et al. 2007, Lewis et al. 2010, Baumann et al. 2011, Smith et al. 2014). Genotypes I and II of

HEV can infect human and non-human primates, whereas humans and pigs are infected by Genotypes III and IV (Balayan et al. 1997). The prevalence of Genotype IV in humans and animals remains unclear, even though Genotype IV of HEV was confirmed to cause zoonosis. The morbidity of this disease in developing countries (Asia and Africa) is higher than that in developed countries (the United States and several European countries) (Meng 2008).

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HEV is a positive-stranded RNA virus, in which three open reading frames, ORF1, ORF2, and ORF3, constitute the coding regions. Non-structural proteins are encoded by ORF1, and are associated with RNA replication (Koonin et al. 1992). For example, the viral methyltransferase is encoded by ORF1 (Huang et al. 2007), and the RNA of the HEV genome was found to have a 5'-cap structure related to the presence of the methyltransferase (Kabrane et al. 1999). HEV vitality and infectivity is maintained because of the cap structure (Magden et al. 2001), which has provided clues for drug discovery to treat HEV. The main coding region is ORF2, which encodes the capsid proteins, possessing viral cellular receptor binding sites (Tam et al. 1991). ORF2 proteins are also considered as the principal target antigen of anti-HEV protective humoral immune responses, because of its many antigenic sites that could produce neutralizing antibodies (Khudyakov et al. 2002). ORF2 has taken center stage in genetic engineering research for vaccines for HEV. Additionally, ORF3 encodes a phosphoprotein that could play a vital role in the specific immune activity of HEV. The phosphoprotein has four confirmed epitopes that might be specific antigenic epitopes (Tsarev et al. 1992).

Cell and tissue culture of HEV are difficult; therefore, traditional inactivated vaccines and attenuated live vaccines are difficult to obtain. To produce an effective vaccine for HEV, the genetic engineering route is the most feasible. Recombinant proteins expressed by insect cells, recombinant proteins expressed in prokaryotic cell and DNA vaccines are candidate vaccines for HEV and have been frequently studied (Mohammad et al. 2011). Protein products expressed by the baculovirus expression system (Luckow et al. 1993) have the advantages of post-translational modification, high biological activity and efficient expression of foreign genes (Ailor et al. 1990, Anderson et al. 1995). Researchers have used baculovirus expression vectors to express the ORF2 protein successfully (Purcell et al. 2003). The results showed that the expressed foreign proteins could induce the production of specific antibodies.

To date, no commercial vaccines for swine HEV have been produced, yet vaccination for this disease is very important. In the present study, a recombinant baculovirus expressing HEV ORF2 was constructed in Sf9 cells using the Bac-to-Bac® baculovirus expression system. Cell lysates of the recombinant baculovirus were used to immunize mice via the oral and intraperitoneal routes to induce humoral and mucosal immunity. The results of the present study lay the foundation for further research and application of swine HEV vaccines.

Materials and Methods

Cell lines and animals

Insect cells (Sf9) were cultured in Grace's medium (Qi Bio-Technique Co. Ltd, Jiang Su, China) containing 10% fetal bovine serum (FBS) (Gibco, Australia) and 1% penicillin-streptomycin at 27°C. 6-week-old BALB/C mice, purchased from Lu Zhou Medical College (Si Chuan, China) were housed separately in specified laboratory room and used in subsequent experiments.

Cloning of swine HEV-ORF2 gene

A fragment of the swine HEV-ORF2 gene region (1198-1881bp) containing crucial neutralizing epitopes was synthesized based on the registered sequences (e.g. GenBank ID: AJ272108 and DQ279091) of swine HEV-ORF2. Synthetic gene fragments were cloned into the pUCK vector and transformed into *Escherichia coli* DH5 α competent cells, and the recombinant plasmid pUCK-ORF2 was synthesized by Shanghai Biological Technology Co., Ltd. pUCK-ORF2 DNA was extracted from the obtained bacterial suspension of *E. coli* cells employing the GV-Plasmid DNA extraction kit (Genview, Beijing, China) and used as template for polymerase chain reaction (PCR) amplification. Specific primers were designed using Primer 5.0 based on nucleotide sequences of the swine HEV genome published in GenBank, as follows: 5'-CCCGGATCCATGTTAATTCGTCGGCTGGTG-3' (Forward primer) 5'-CCCGTTCGACCGTGTCCTCCAAAACAGCCAG-3' (Reverse primer). BamH I and Sal I restriction sites were included in the primer. PCR amplification was performed for 30 cycles using the following program: Pre-denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. Afterwards, the recombinant plasmid was subjected by sequencing.

Construction of a recombinant transfer vector

Plasmid pUCK-ORF2 and the baculovirus expression vector pFastBac™ 1 were subjected to restriction digestion with BamH I and Sal I, then ligated together at 16°C for 12-16 h to construct the recombinant transfer vector pFastBac™ 1-ORF2. Recombinant vector pFastBac™ 1-ORF2 was transformed into *E. coli* DH5 α competent cells, extracted with the GV-Plasmid DNA extraction kit (Genview), and digested with BamH I and Sal I for identification.

The verified positive plasmid sequence was confirmed by sequencing as described above.

Construction of a recombinant Bacmid vector

The recombinant vector pFastBac™ 1-ORF2 was transformed into *E. coli* DH10Bac competent cells and cultured on Luria-Bertani (LB) agar plates containing Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma, St. Louis, MO, USA), X-gal, gentamicin, kanamycin and tetracycline in the dark at 37°C for 48 h. The recombinant colonies were transferred into LB liquid medium containing gentamicin, kanamycin, and tetracycline at 37°C with shaking overnight. The shuttle plasmid was extracted using a GV-plasmid DNA mini Extraction kit (Genview). The recombinant Bacmid-ORF2 was identified by PCR amplification using M13 primers as follows: 5'-CCCAGTCACGAC-GTTGTAACACG-3' (Forward primer) 5'-AGCGGATAACAATTTACACAGG-3' (Reverse primer). The following program was for PCR amplification: 30 cycles of pre-denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 3 min 30 s; and extension at 72°C for 10 min. Finally, the sequence of positive plasmid rBacmid-ORF2 was confirmed by sequencing as described above.

Acquisition of recombinant baculoviruses

To obtain the recombinant baculoviruses, bacterial supernatant from the previous step was incubated at 37°C with shaking overnight. Based on the manufacturer's instructions of Bac-to-Bac® Baculovirus Expression system, Sf9 cells were seeded onto sterile 6-well plastic plates and transfected with positive rBacmid-ORF2 using Cellfectin II Reagent at 27°C for 72 hours or until a significant cytopathic effect of viral infection appeared in the Sf9 cells. Budded virus was regarded as the P1 viral stock that could be released into the medium and harvested from the cell culture after three repeated freeze-thaw cycles that was named the recombinant baculovirus rBacmid-ORF2 P1. PCR and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) were used to identify the recombinant baculovirus.

Analyzing the recombinant baculovirus by PCR and SDS-PAGE

Total viral RNA was extracted from the recombinant baculovirus rBacmid-ORF2 using the RNAiso

plus reagent (Takara Biotechnology, Dalian Co., Ltd., China) following the manufacturer's instructions. Reverse transcription PCR (RT-PCR) was conducted using an RNA PCR™ Kit (AMV) Ver. 3.0 (Takara Biotechnology). PCR amplification was performed using the following thermocycler program: 30 cycles of pre-denaturation at 94°C for 3 min, denaturation at 94°C for 30s, annealing at 55°C for 30 s, extension at 72°C for 3 min 30 s; and extension at 72°C for 10 min using M13 primers. Additionally, the P1 viral stock of rBacmid-ORF2 was used to infect Sf9 cells for 72 h to generate a high-titer P2 stock, which in turn was used to obtain rBacmid-ORF2 P3. The harvesting methods for wild-type P3 were the same as those for rBacmid-ORF2 P3. Separately, Bacmid-ORF2 P3 and wild-type P3 were cultivated in Sf9 cells at 27°C and 5% CO₂ for 72 h. After three repeated cycles of freeze-thawing, the supernatant and precipitate from the cell culture were collected by high speed centrifugation and added to 5 × SDS loading buffer (Beyotime Biotech Co., Ltd., Shanghai, China) for SDS-PAGE detection.

Detection of mouse antibody levels using an enzyme-linked immunosorbent assay (ELISA)

Four groups of mice (oral, intraperitoneal injection, negative control, and the blank control) were treated with rBacmid-ORF2 cell lysates via the oral route, rBacmid-ORF2 cell lysates via intraperitoneal route, wild-type baculovirus cell lysates, and PBS buffer (pH = 7.0) via the oral route, respectively, with 100 μL per mouse. Immunization was performed at 0, 14, and 28 days. Serum and feces were collected separately at 0, 14, 28, and 42 days. Serum samples were collected through tail bleeding to test for swine HEV IgG antibody levels using a swine HEV antibody-IgA ELISA kit (Kexing Biotech, Shanghai, China Co., Ltd.). Fecal samples were collected in sterile PBS (pH = 7.0) to detect swine HEV SIgA antibody levels using a swine HEV antibody-IgA ELISA kit, following the manufacturer's instructions. A microplate reader was used to detect the OD₄₅₀.

Statistical analysis

The experimental results are shown as the mean ± SEM of the three independently repeated experiments. The data from all results for the antibody levels were analyzed statistically using one-way analysis of variance (ANOVA) for any difference between the four groups of mice (oral, intraperitoneal injection, negative control, and blank control). The least

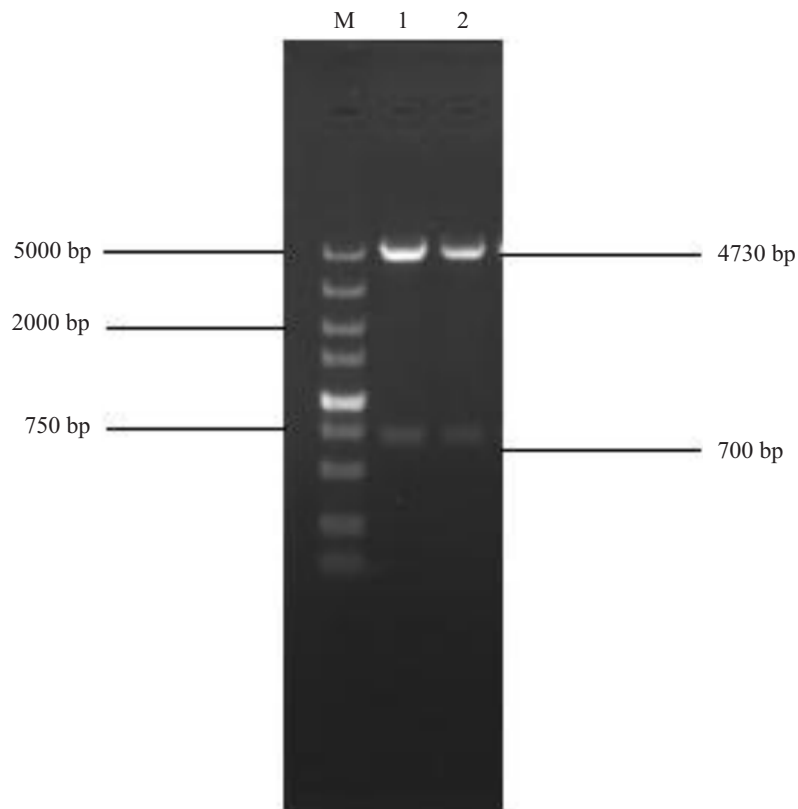


Fig. 1. Identification of pFastBac™ 1-ORF2 digested by restriction enzymes. (M) Marker (DL-5000). Lanes 1 and 2 are pFastBac™ 1-ORF2 digested by BamH I and Sal I, respectively.

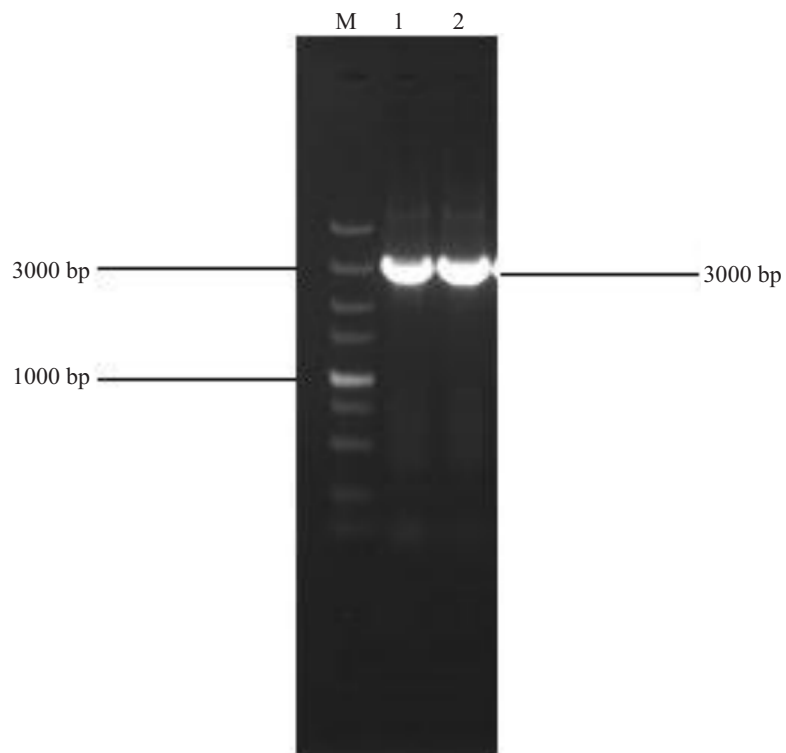


Fig. 2. Fragment of M13 amplified using PCR. M, Marker (DL-5000). Lanes 1 and 2 are the M13 fragments generated by PCR.

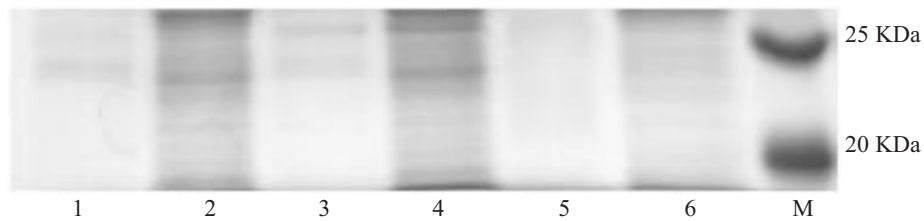


Fig. 3. Sodium dodecylsulfate polyacrylamide gel electrophoresis of recombinant protein rBacmid-ORF2. Lane 1, supernatant of wild-type baculovirus. Lane 2, precipitate of wild-type baculovirus. Lane 3, supernatant of rBacmid-ORF2. Lane 4, precipitate of rBacmid-ORF2. Lane 5, supernatant of normal Sf9 cells. Lane 6, precipitate of normal Sf9 cells. Lane M, protein marker. Recombinant baculovirus rBacmid-ORF2 was identified with a molecular masses of 26 KDa.

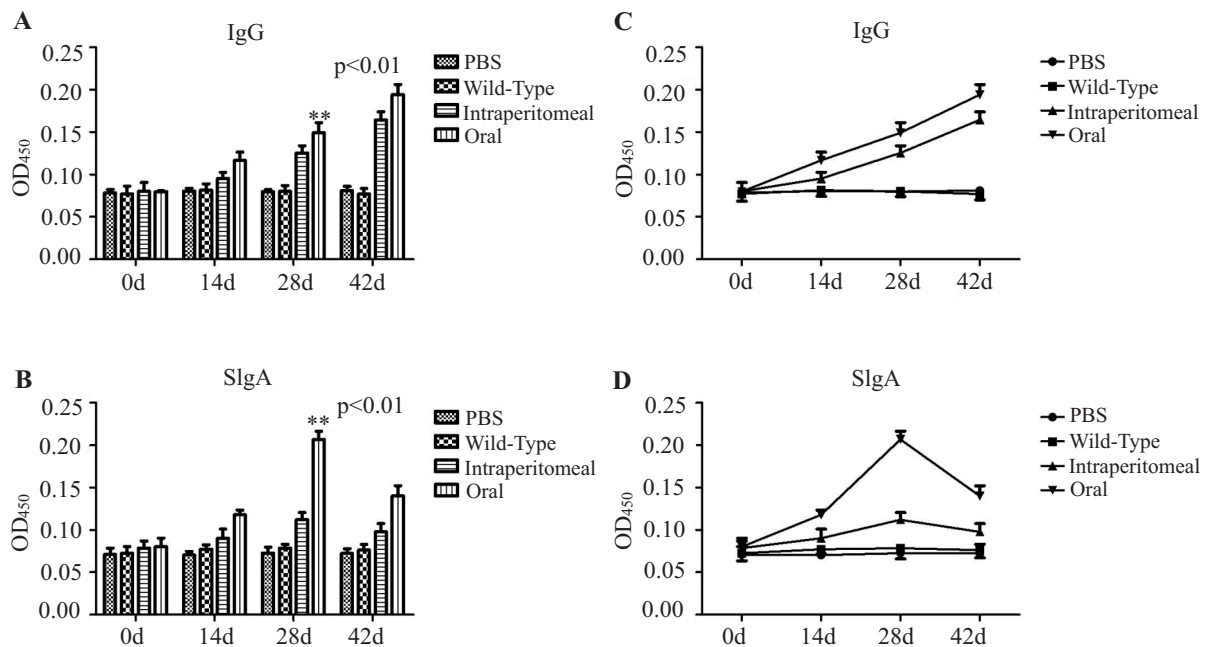


Fig. 4. Enzyme-linked immunosorbent assay (ELISA) of hepatitis E virus (HEV). (A,C) ELISA detection of swine HEV IgG antibody levels in mice after immunization with recombinant baculovirus rBacmid-ORF2. Serum collected at different times after immunization in mice to check IgG levels in four groups (immunized with phosphate-buffered saline (PBS), wild-type virus, rBacmid-ORF2 administered intraperitoneally, and rBacmid-ORF2 administered orally). (B,D) ELISA detection of swine HEV SIgA antibody levels in mice after immunization with rBacmid-ORF2. Mice feces collected at different times after immunization were checked for SIgA levels in the four groups. The experimental results were displayed as the mean \pm SEM of three independently repeated experiments. $p < 0.01$ were regarded as the determinant levels of statistical significance. (One-way ANOVA, LSD tests).

significant difference (LSD) test was used for multiple comparisons. All statistical analyses were performed using the SPSS 20.0 software. p values < 0.05 or < 0.01 were considered significant and very significant, respectively.

Results

Construction of recombinant transfer vector (pFastBacTM 1-ORF2)

pFastBacTM 1 and pUCK-ORF2 were digested to construct the recombinant transfer vector pFastBacTM 1-ORF2. Correct recombinants were identified by

PCR and dual enzyme digestion, and a 700 bp fragment of PCR product was observed that was consistent with HEV ORF2 gene region. Electrophoresis results also showed the expected bands at 4730 bp and 700 bp after digestion, suggesting that the recombinant transfer vector (pFastBacTM 1-ORF2) was constructed successfully (Fig. 1).

Construction of a recombinant Bacmid vector

Correctly identified pFastBacTM-ORF2 was transferred into competent DH10BacTM *E. coli* cells. The PCR electrophoresis result of the extracted recombinant shuttle plasmid Bacmid-ORF2 showed the expected 3000 bp fragment as a clear band of the correct

molecular weight, with no non-specific amplification (Fig. 2).

Identification of recombinant baculovirus

Positive plasmid Bacmid-ORF2 was transfected into Sf9 insect cells to obtain recombinant baculovirus rBacmid-ORF2. The extracted RNA of rBacmid-ORF2 was detected by RT-PCR. For each group, the supernatant and precipitate from Sf9 insect cells infected by recombinant baculovirus were used for SDS-PAGE analysis. Figure 3 shows that clear bands of 26 kDa were found in the precipitate of the recombinant virus. The results suggested that the recombinant protein was expressed correctly in the baculovirus expression system.

Humoral immune responses induced by recombinant baculovirus harboring HEV-ORF2

Serum was collected from immunized mice at 0, 14, 28, and 42 d to detect IgG antibody levels by ELISA, representing the virus-induced humoral immune response. The results indicated that there was no significant difference in IgG antibody levels between the groups of PBS and wild-type baculovirus after immunization at each time point. The IgG antibody levels of the oral and intraperitoneal immunization group showed sustained increases in IgG levels from 14-42 d, which were significantly higher than those produced by the PBS and wild-type baculovirus groups ($p < 0.01$). Relatively high IgG levels were produced in the oral group (Fig. 4 A, C).

Mucosal immune responses induced by recombinant baculovirus harboring HEV-ORF2

Feces were collected from immunized mice at 0, 14, 28, and 42 d to detect SIgA antibody levels by ELISA, representing the virus-induced mucosal immune response. The results indicated that there was no significant difference in SIgA antibody levels between the groups of PBS and wild-type baculovirus after immunization at each time point. At 14 d, the SIgA levels of the oral group were very significantly higher than those in the other three groups. The intraperitoneal and wild-type baculovirus groups also showed increased SIgA levels; however, the levels in the intraperitoneal group were significantly higher than those in the wild-type baculovirus group

($p < 0.05$). At 28 d, the SIgA levels in the oral and intraperitoneal groups were very significant higher than those in the other groups ($p < 0.01$); however, there was no significant difference between the wild-type baculovirus and PBS groups. Between 28 and 42 d after immunization, the antibody levels in the oral and intraperitoneal groups decreased; however, at 42 d, the level of SIgA in the oral group remained very significantly higher than that in the other groups ($p < 0.01$) (Fig. 4B, D).

Discussion

Clinical studies have shown that acute hepatitis can be caused by HEV. Younger people are primarily infected and a higher mortality rate was observed in pregnant women infected by HEV. There is a high morbidity rate in China and HE cases show an upward trend. The prevention and treatment of HE infection are of great significance to human health and animal industries. The encoded protein of ORF2 was the main object of this research to genetically engineer an HEV vaccine because of its numerous antigen epitopes. Recombinant baculovirus HEV nucleoproteins expressed in insect cells have been regarded as the satisfactory vaccine candidates. We used fragments from the ORF2 region in a baculovirus expression system to construct and express recombinant baculovirus for swine HEV ORF2.

In this experiment, amplified target fragment of swine HEV ORF2 was inserted into promoter downstream of transfer vector pFastBac™ 1PH. The left arm and right arm of Tn7 are located on the two sides of the multiple cloning site to form a micro-Tn7 unit. With the aid of helper plasmids, the micro-Tn7 unit is combined with target sites of mini-attTn7 to construct recombinant shuttle plasmids after the recombinant transfer plasmids were transformed into *E. coli* DH10Bac competent cells, based on the manufacturer's instructions of the Bac-to-Bac® Baculovirus Expression System. Recombinant bacmid DNA could only be identified by PCR because of the fragment length greater than 135 kb restriction enzymes cannot be used for identification. The universal M13 primer matched with the target site of mini-attTn7 and was selected to verify via PCR if the target fragment was inserted correctly. The constructed Bacmid-ORF2 was transfected into Sf9 insect cells to obtain recombinant baculovirus and was stably amplified in the amplification culture. Supernatant and precipitate of recombinant baculovirus, wild baculovirus, and common Sf9 cells were analysed by SDS-PAGE. The results suggested that ORF2 protein was expressed from the baculovirus successfully.

Previously, animals were immunized with Swine HEV structural proteins expressed by a baculovirus via the parenteral intramuscular injection and intraperitoneal routes to produce specific IgG antibodies. Animals inoculated with the recombinant proteins only induced antibodies at a low titer or produced no IgA, and thus could not produce an effective passive immunity for the animals (Mohammad et al. 2011).

In the present study, recombinant baculovirus ORF2 protein of swine HEV was administered via oral and intraperitoneal routes to explore levels of antibodies induced in mice. The ELISA results for IgG and IgA to Swine HEV indicated that mice immunized using the recombinant baculovirus could induce a humoral response via both routes, but only oral delivery induced significant mucosal immunity. The reasons could be related to the antigenic quality of baculovirus, the types of immune response, and the response levels. The mucosal immune response induced by the intraperitoneal route approach required higher doses of the virus to produce high levels of IgA antibodies.

Conclusion

In conclusion, the ORF2 region of HEV is rich in antigenic epitopes, and was chosen to construct a recombinant baculovirus of swine HEV ORF2. Mucosal and humoral immune responses were induced by immunization via the oral and intraperitoneal routes in mice. This preliminary research suggested that mucosal and humoral immunity could be induced using baculovirus as the carrier to deliver foreign genes, representing a new approach to produce gene vaccines for swine HEV.

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