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Original Research

Prediction of G gene epitopes of viral hemorrhagic septicemia virus and eukaryotic expression of major antigen determinant sequence

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Abstract: This study aims to express fish Viral hemorrhagic septicemia virus (VHSV) G main antigen domain by using Bac-to-bac expression system. Using bioinformatics tools, B cell epitope of VHSV G gene was predicted, and G main antigen domain was optimized. GM gene was inserted into pFastBac1 vector, then transferred recombinant plasmid into DH10Bac to get recombinant rBacmid-GM. Obtained shuttle plasmid rBacmid-GM was transfected into sf9 cells. GM expression was examined using by PCR and western-blot. Results indicated that G main antigen domain gene of VHSV was successfully cloned and sequenced which contains 1209 bp. PCR proved that shuttle plasmid rBacmid-GM was constructed correctly. SDS-PAGE electrophoresis analysis detected a band of protein about 45kD in expression product of G gene. Obtained recombinant G protein reacted with VHSV-positive serum that was substantiated by western-blot analysis. In conclusion, the main antigen domain of VHSV G was successfully expressed in the Bac-to-Bac baculovirus system.

Key words: Viral haemorrhagic septicaemia virus; G gene; Eukaryotic expression.

Introduction

Viral hemorrhagic septicemia virus (VHSV), a member of the family Rhabdoviridae and genus Novirhabdovirus (1), is the main cause of the epidemic outbreak of fulminating infectious disease in salmons and various marine fish. The genome of this virus consists of six genes successively from the 3' end to 5' end, namely, N (nuclear protein)-P (Phosphoprotein)-M (matrix protein)-G (glycoprotein) -NV (non-structural protein)-L (polymerase protein).

Surface glycoprotein G is one of the important antigens, and it is also a preferred material for antibody preparation in various immunological tests (2). However, the yield of unmodified wild-type G gene expressed in vitro is low due to the codon preference in different species (3,4), which prevents the production of the antibody against the VHSV G gene-encoded protein.

The study was designed to obtain a recombinant protein with a spatial structure more close to VHSV G protein in the eukaryotic expression system (5-7), laying a good foundation for further study of the function of G protein. But the eukaryotic expressions of G gene in our previous study were failed. For this reason, the strategy of multi-parameter prediction of B-cell epitopes in G protein combined with the expression of truncated main antigenic domain was taken in this study, and this main antigenic domain of G protein was efficiently expressed in the Bac-to-Bac baculovirus expression system. The immunogenicity of the expressed protein was verified in the preliminary identification and analysis of the activity by Western blotting, serving as a basis for the further development of epitope vaccine and the establishment of molecular diagnostic technique for VHSV.

Materials and Methods

Strains, cell lines, and plasmids

VHSV-H strain was kindly provided by Shenzhen Entry-Exit Inspection and Quarantine Bureau. E.coli BL21, transposon plasmid pFastBac1, and DH10Bac™ competent cells were supplied by GenScript (Nanjing) Co., Ltd.. Sf9 insect cells were provided by Qingdao Vland Biological Products Co.,Ltd., and myeloma cells SP2/0 was preserved by our laboratory.

Reagents

Fetal bovine serum (FBS), Cellfectin II Reagent, insect cell culture medium sf900- II SFM, Grace medium, and Lipofectaminetm reagent were purchased from Gibco company; Razoal RNA extraction kit was purchased from Promega company; M-MLV reverse transcriptase, Ex Taq DNA polymerase, and restriction endonucleases were purchased from TaKaRa company; T4 ligase and RNA extraction kit were purchased from Promega company; gel extraction kit and Plasmid Extraction Kit were purchased from Tiangen company; 6 X His protein purification resin Ni-NTA was purchased from QIAGEN company; rabbit anti-goat HRP-IgG and goat anti-mouse HRP-IgG were purchased from Jackson company; goat anti-mouse FITC-IgG, PEG4000, and HAT were purchased from Sigma company; BALB/c mice were purchased from the experimental animal center of Shandong University; and goat anti-VHSV polyclonal serum was kindly provided by Chinese Academy of inspection and quarantine.

Prediction of main antigenic domains using bioinformatics analysis tools

The NCBI Conserved Domains search tool was employed to analyze the conserved domains in the amino acid sequence of VHSV G protein, and signal peptide sequence of this protein was predicted by using the online program SignalP4.1 on the website of the center of biological sequence analysis (CBS) of University of Denmark. The tertiary structure of the protein was analyzed with the Phyre program in online system http://www.sbg.bio.ic.ac.uk/servers/phyre. OptimumAntigen™ software was used to predict the dominant antigenic peptide, and Jameson-Wolf (8) method, which combines 4 parameters of hydrophilicity, surface properties, flexibility, and secondary structure together, was utilized to comprehensively predict the antigenic index of this protein. And based on the results, main antigenic domain (MAD) was chosen and named GM.

Synthesis of the codon-optimized gene

By using the codon analysis software on http://gcua.schoedl.de/ server, codon frequency of VHSV-H gene sequence (GenBank accession number KJ768664) in insect cells were analyzed, and it was found that the codon frequency of 17 codons, totally 85 sites, was lower than 20% in insect cells. Synonymous mutations were performed for 64 sites of low-frequency codons in the densely located regions in the G gene, and a restriction site linker (EcoR I and Hind III) and a histidine tag sequence (6his) were added at the C terminal of the MSD of the G gene for the convenience of purification, which was named GM. The sequence of GM was submitted to GenScript (Nanjing) Co., Ltd., where the gene was synthesized and cloned into pUC57.

Construction and identification of recombinant plasmid pFastBac1-GM

Plasmid pUC57-GM and transposon plasmid pFsatBac1were double digested with EcoR I and Hind III. These digested products were recovered after gel extraction, ligated with T4 DNA ligase, and then transformed into BL21 competent cells. Following the resistance screening by ampicillin, single colonies were picked, from which plasmids were extracted and digested with EcoR I and Hind III, to identify the positive recombinant plasmids.

Transposition of recombinant plasmid pFastBac1-GM and identification of the recombinant Bacmid

The recombinant plasmid pFastBac1-GM was transformed into DH10Bac competent cells containing Bacmid. White colonies were picked by the resistance screening with three antibiotics (gentamicin 7μg/mL, kanamycin 50μg/mL, and tetracycline 10μg/mL) and the blue-white selection. PCR amplification for the recombinant Bacmid was performed using universal primers for M13F/M13R (upstream : 5'-CCCAGTCACGACGTTGTAAAACG-3', and downstream : 5'-AGCCCATAACAATTTCACACAGG-3'), and the amplification conditions were as follows: pre-denaturation, 93°C, 3mins; 94°C, 30s, 55°C, 45s, and 72°C 2mins, for 30 cycles; and 72°C, 7mins. Positive shuttle plasmid identified was named rBacmid-GM. PFastBac1 plasmid was treated in the same way, and the obtained rBacmid-N served as the negative control.

Transfection of recombinant Bacmid and identification of the product expressed

The recombinant rBacmid-GM and rBacmid-N were transfected into Sf9 insect cells (9x 10^5/ mL) by liposome method, and the cells were cultured at 28°C. The supernatant of cell culture was collected when cellular pathological changes could be observed. The transfected Sf9 cells were sub-cultured for 3 generations.

Western blot

After 3 passages, the transfected cells were collected and lysed with cell lysis buffer. The supernatant was removed and loaded for SDS-PAGE electrophoresis. As a negative control, the normal Sf9 cells were treated with the same method. The proteins were separated by SDS-PAGE electrophoresis and transferred to PVDF membrane, which was then blocked with 3% BSA. The membrane was incubated with goat anti-VHSV polyclonal serum diluted at a ratio of 1:100 , at 37°C for 1hr, and then rabbit anti-goat HRP-IgG diluted at 1:2000 was added and incubated at 37°C for 1hr, followed by the chromogenic reaction and identification at the final step.

Results

Search for the structural domains of G protein

The NCBI Conserved Domains search tool was used to analyze the amino acid sequence of the G protein, and 1 conserved domain was found, which is a member of the capsid glycoprotein family in rhabdovirus.

Prediction of the signal peptide of G protein

Signal peptide sequence in the amino acid sequence of G protein was predicted using the online program SignalP4.1 on the website of the center of biological sequence analysis (CBS) of University of Denmark, and the result was shown in Figure 1. It can be predicted in the graph that there was a signal peptide cleavage site between aa sites 20-21, indicating the exixtence of a signal peptide in the VHSV G protein sequence. Therefore, this signal peptide was removed or avoided in the subsequent expression.

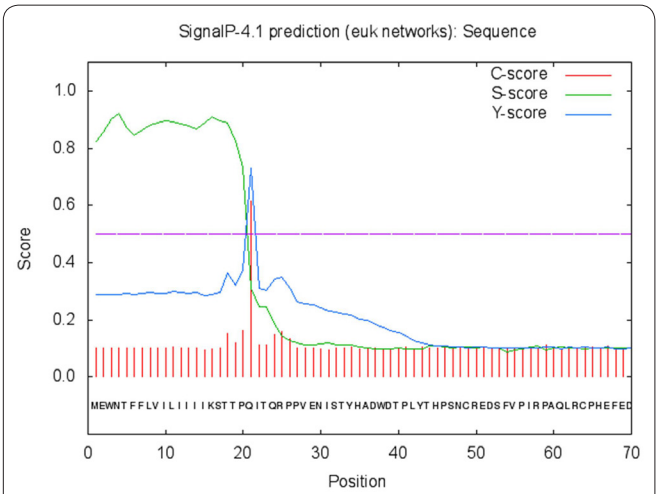


Figure 1. Result of singalP-NN prediction on VHSV G deduced amino acid sequence.

Table 1. antigenic epitopes analysis of VHSV-G protein.

No	Start	Antigenic Determinant	Length	Antigenicity/Surface/ Hydrophilicity	Disordered Score	Synthesis	Mus musculus | Oryctolaguscuniculus blast
1	274	CPGGARKLTPKKCVN	14	2.58/0.79/0.54	0.1443	Hard	63% | 49%
2	131	RQEATNEASKDHEYC	14	2.56/0.79/0.85	0.1053	Easy	49% | 56%
3	44	THPSNCREDSFVPIC	14	2.14/0.71/0.22	NONE	Hard	49% | 57%
4	333	CLSKFRPSHPGPGKA	14	1.92/0.79/0.10	NONE	Easy	56% | 49%
5	17	STTPQITQRPPVENC	14	1.92/0.79/0.11	0.1219	Easy	56% | 64%
6	314	CECLDAHSDITASGK	14	1.82/0.64/0.29	NONE	Hard	42% | 78%
7	218	CSETLEGHLFTRTHD	14	1.70/0.50/0.15	NONE	Easy	64% | 49%
8	383	CSWKRVNNNTDGYDG	14	1.53/0.71/0.29	NONE	Hard	42% | 50%
9	158	NVHKDITHYYKTPKC	14	1.52/0.71/0.13	NONE	Easy	50% | 42%
10	62	LRCPHEFEDINKGLC	14	1.42/0.50/0.38	NONE	Hard	49% | 50%
11	348	CYLLEGQIMRGDCDY	14	1.10/0.43/0.21	NONE	Hard	42% | 56%
12	179	SRKFLNPDFIEGVC	14	1.07/0.64/-0.03	NONE	Easy	57% | 42%
13	402	CKLIIPDIEKYQSVY	14	1.05/0.57/0.17	NONE	Easy	57% | 64%
14	291	CQMRGATDDFSYLNH	14	0.97/0.79/-0.05	NONE	Easy	64% | 64%

Epitope prediction of protein G

The OptimumAntigen™ software was used to predict the epitope of protein G, and the result showed that the antigenic indexes of most regions of protein G in this strain were relatively high. Although these regions were located in the hydrophilic and plasticity regions, it was generally believed that the region where an epitope may locate must be a solvent accessible one. The amino acid sequence in the blue region showed a high antigenic index and could be the dominant region of the epitope on B cell (Table 1 and Figure 2). So peptide between amino acid sites 40-435 was selected and this region with the highest antigenic index was used in the subsequent expression while avoiding the signal peptide.

Result of plasmid pFastBac1-GM identification

The recombinant plasmid was double digested with EcoR I/Hind III, and the results showed that the transposon plasmid pFastBac1-GM was successfully constructed (Figure 3A).

Result of recombinant Bacm identification

The recombinant plasmid pFastBac1-GM was transformed into DH10Bac competent cells and screened with three antibiotics and blue-white selection. PCR method was employed in the identification of the recombinant plasmid by using M13F/M13R primers. There was a specific band with a size of 3500bp on agarose gel, demonstrating the successful construction of the recombinant plasmid rBacmid-GM (Figure 3B).

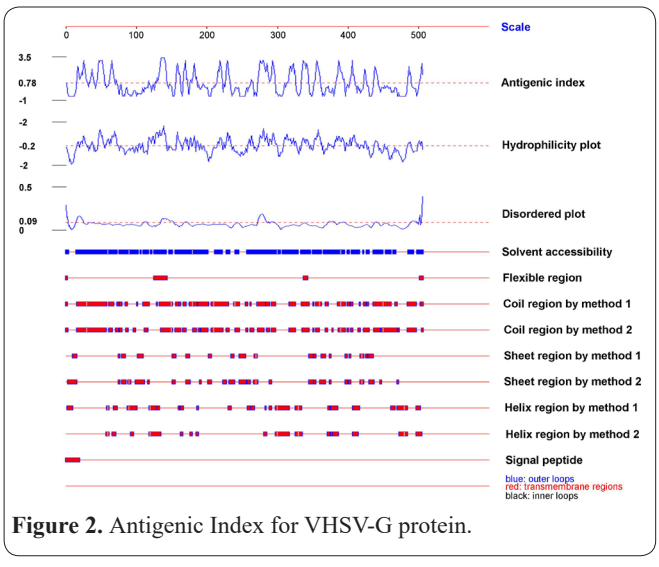


Figure 2. Antigenic Index for VHSV-G protein.

Transfection of the recombinant Bacmid into sf9 insect cells

The recombinant Bacmid was transfected into sf9insect cells, and pathological changes were observed 5 days after the transfection (Figure 4A). After a series of passages, the lesions were obviously enhanced, which was shown as cell enlargement, cessation of cell division and proliferation, enlarged nucleus filling the whole cytoplasm, and inclusion body like particles in the nucleus. More cells detached and started to lyse with the infection time prolonged. The titer of the second generation virus was 3.37×10^8 pfu/ml.

Transfection, virus identification, and expression analysis

The recombinant baculovirus was inoculated into sf9

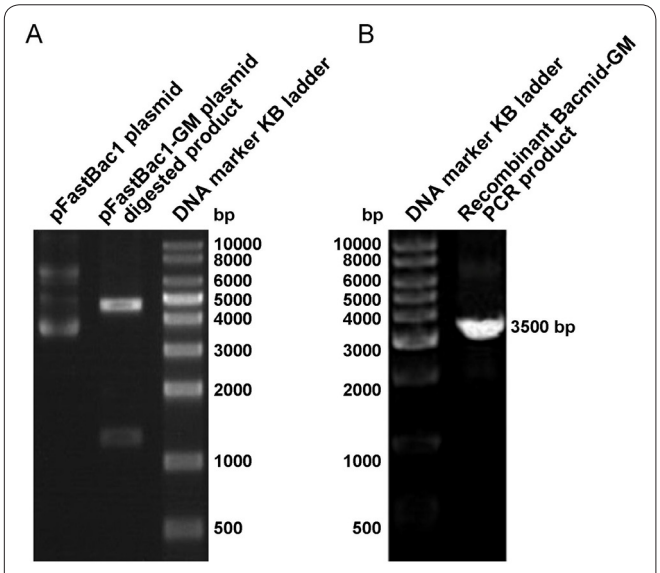


Figure 3. Electrophoresis identification of recombinant plasmid with restriction enzyme and recombinant plasmid rBcmid-GM. A. Identifiction of recombinant plasmid. B. Identification of recombinant plasmid rBcmid-GM by PCR.

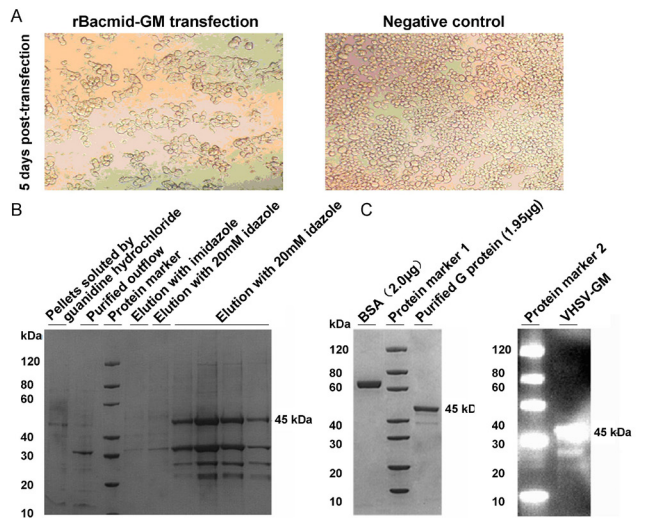
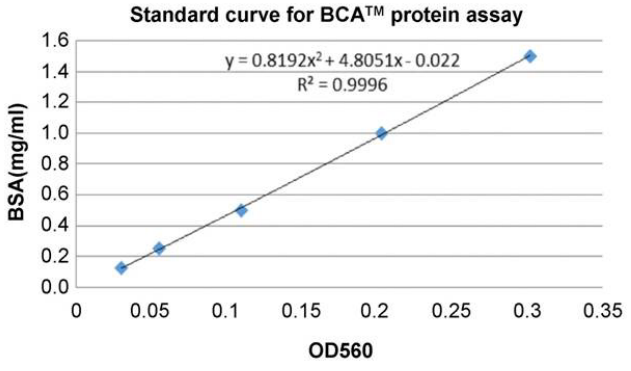


Figure 4. Pathological changes of transfected sf9 and the purification and identification. A. Pathological changes of transfected sf9 cells (×100). B. The supernatant and precipitated pellets were purified by Ni column. C. Western blot analysis for the Ni column purifed protein. Protein marker 1 includes the molecule weight of 120, 80, 60, 40, 30, 20 and 10 bp, upwards to downwards. Protein marker 2 includes the molecule weight of 120, 85, 60, 40 and 22 bp, upwards to downwards.

cells, and harvested after 72 hours. The collected viruses were lysed with lysis buffer containing protease inhibitor, followed by ultrasonication. Then the lysate was centrifuged. The supernatant and precipitated pellets were purified by Ni column, and the different fractions were analyzed by SDS-PAGE. The results (Figure 4B) revealed an expected band with the size of about 45kD. Western blotting result showed a brown reaction band at the site of the target protein, indicating that His-GM was recognized by goat anti-VHSV polyclonal serum (Figure 4C). BCA™ method using BSA as a standard reference was employed to determine the concentration of protein, which was up to 0.4mg/mL (Figure 5).

Discussion

The VHSV stain isolated and preserved in our lab belongs to the Ib subfamily based on the full length sequence of G gene, and it can infect at least 10 species



Sample	OD value: 560 nm	Concentration
VHSV-G	0.087	0.40 mg/ml

Figure 5. Evaluation for protein concentration by using BCA™ method.

of fish in the Baltic Sea and Beihai sea, causing an exotic epidemic outbreak of infectious disease in aquatic animals. At present, the blocking and early warning monitoring methods in China are those used to determine the pathogens, and then suspected positive samples are confirmed by RT-PCR. However, the PCR method can only be used as a preliminary diagnosis, so immunological methods are urgently needed as supplementary diagnoses. Currently, all of the VHS viral genotype and serum type can react with the monoclonal antibody IP5B11 (9-11), which should be purchased from abroad. In addition, the antigenicity across different species of rhabdovirus is similar, and the specificity of polyclonal antibodies can't meet the requirements. Therefore, it is urgent to independently develop a universal VHSV monoclonal antibody in China.

Lorenzen et al. (12) purified the VHSV by differential centrifugation, and using it as an immunogen, three monoclonal antibodies against VHSV were screened out. However, to do this, a large number of viruses are needed and multiple purifications are repeated, which is a complicated process. The Bac-to-Bac baculovirus expression system invented by Luckow and colleagues is a technique that takes the advantage of the bacteria transposon, and can be used in the rapid construction of recombinant virus in Escherichia coli. Compared with the traditional method, the system can be employed in Escherichia coli and the recombinant virus can be directly infected into insect cells, greatly reducing the time for the construction of a recombinant baculovirus. Meanwhile, the blue-white screening is used in the selection of the recombinant vector, with no issues such as the cross contamination between the wild-type and the non-recombinant virus. More cycles of plaque assays are not necessary to purify the recombinant viruses, which are helpful in the screening of recombinant viruses. In this study, this system was selected and positive recombinant Bacmid was directly obtained through the resistance screening with three antibiotics, leaving out the plaque purification process. And the expressed recombinant protein, carrying the 6 x His histidine tag, makes the protein purification simple and easy.

Paloma et al. (13) demonstrated in massive production of VHSV gpG protein that the fragment of G protein (21aa-465aa) was highly expressed when the transmembrane region and the carboxy terminal of the protein were both removed. Considering that the signal peptide sequence of G protein may not be recognized and cleaved in insect cells (14,15), the signal peptide of G protein was predicted and avoided in this study, and meanwhile, the OptimumAntigen™ software was used to predict the epitope of this protein, and the peptide fragment between amino acid sites 40-435 was selected for the codon optimization and gene synthesis. The concentration of G protein produced in this system could reach 0.4mg/mL. Protein identification results showed that the protein could be recognized by goat anti-VHSV polyclonal serum, which is consistent with the expected findings of the present study. Moreover, there also many other studies (16-19) have been reported the vaccines that against the viral hemorrhagic septicemia virus by targeting the glycoproteins in fish. However, it's also controversial for the mechanism for the immune responses to the bacterial and viral infections, which

would be investiaged in the following study.

In conclusion, the main antigen domain of VHSV G was successfully expressed in the Bac-to-Bac baculovirus system.

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