Construction of Recombinant Rabbit Hemorrhagic Disease Virus (RHDV) Vaccine using Myxoma Virus (MV) as a Vector

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Abstract—**Rabbit hemorrhagic disease virus (RHDV) and myxoma virus (MV) are the two main viruses that cause serious virus diseases in rabbit population. MV, a pox virus, has been proved to be a good recombinant vaccine vector. In this study we have developed a recombinant virus using MV as a vector against both the myxomatosis and rabbit hemorrhagic disease (RHD). We used the nonessential gene MST3N of MV as the insertion site. The recombinant viruses expressed the RHDV major capsid protein (VP60) and the selectable marker GFP. The pure recombinant viruses were achieved after several rounds of plaque screening. Replication of MST3N-knockout recombinant virus MV-VP60 in Rabbit kidney cell (RK13) was unimpaired, compared with wild type virus MV.**

Keywords-Rabbit hemorrhagic disease virus (RHDV), tmyxoma virus (MV), non essential MST3N gene, vector.

I. INTRODUCTION

Rabbit Hemorrhagic disease (RHD) and Myxomatosis are considered to be the major viral diseases affecting European rabbit population. The RHD which caused by Rabbit Hemorrhagic disease virus (RHDV) is an acute and highly contagious disease in wild and domestic rabbits[1]. Infected rabbits usually die within 48 to 72 h of necrotizing hepatitis. The disease is responsible for high economic losses in rabbitries as well as high mortality rates in wild rabbit population. The disease was first reported in China in 1984 and then also found in other parts of the world. The disease attracted a wide attention for its high rates of death and transmission[2]. The RHDV is a member of the genus *Lagovirus*, family *Caliciviridae*. The virion contains a 7.4Kb single-stranded positive-sense RNA genome. The capsid consists of a major protein component of 60 kDa (Vp60)[3] . Myxoma virus (MV), whose genome composes of 163kb nucleotides, belongs to the *Leporipoxvirus* genus of the *Poxviridae* family. This virus replicates in the cytoplasm of infected cells, the typical characteristic of poxvirus. The virus induces a benign disease in its natural host, Sylviagus rabbits in the Americas. In European rabbits, however, MV causes myxomatosis, a systemic and usually fatal disease. This disease only occurs in wild and domestic rabbits and other animals and human are not susceptible to this virus. The virus is usually transmitted by blood-feeding arthropod vectors such as mosquitoes or fleas [4].

In this study, we used the MV as a recombinant vector for delivery of the structural gene VP60 of RHDV which served as epitope for antibody. First, we constructed a transfer vector by flanking the VP60 gene of the RHDV and a selection marker GFP within a non-essential gene MST3N gene of MV. In this construction, both the VP60 and GFP genes with opposite orientation are under the control of the 7.5 promoter of the vaccinia virus. MV BE4 virus strain with an appropriate virulence was selected as the recombinant virus vector. The RK13 cells initially infected with wild-type BE4 virus were transfected with recombinant plasmid pMVP60. Based on homologous recombination between the MST3N gene of the wild-type MV and that of the transfer vector, recombinant MVs were selected and picked up in the plaques containing GFP marker using the Fluorescence microscopy after infection of RK13 cell monolayer with the supernatant of pMVP60 transfected RK13 cells. The expression of RHDV VP60 by recombinant virus was confirmed by Western blot.

II. MATERIALS AND METHODS

A. Cells and viruses

Rabbit kidney-13 (RK13) cells were grown in RPM1640 supplemented with 10% fetal bovine serum (Gibco-BRL). MV BE4 was isolated in the Grande Motte in France and maintained by Bio Espace.

B. construction of recombinant transfer vectors

The procedure used to construct recombinant MV viruses was adapted from standard methods previously described for vaccinia virus (VV) [5]. A transfer vector pMVp60a, with MV DNA flanking regions suitable to direct foreign gene insertion into MV genome by homologous recombination, was constructed as follows. Two MV DNA fragments from the intergenic site of MST3N gene were amplified by PCR using oligonucleotide primers: F1 5'-TAACC GGTACC CGA ATCCCGTAATCGTTA-3' (KpnI site underlined), and F2 5'-TTATA CTCGAG GACTCGTACGACGTGGTGTT-3' (*XhoI* site underlined) for the left flank sequence (406 bp) and oligonucleotide primers: F3 5'-AATAT CGGCCG CGTACGAGTCGATGATTCGT-3' (Bstz*I* site underlined *BamHI* site in boldface) and F4 5'-TATAC GAGCTC

# *Zhen Guo and Qian Yu contributed equally to this work*
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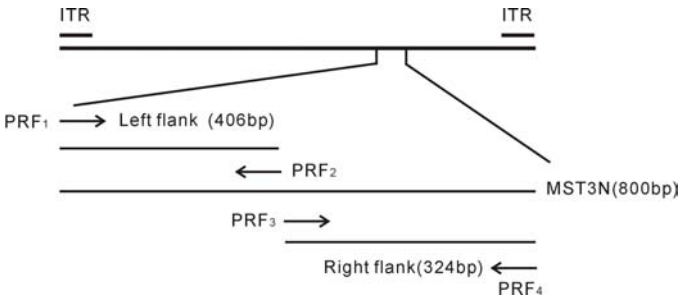
GATGCACGATGGTGGTGTACGCA-3' (*SacI* site underlined) for the right flank (324 bp). After PCR amplification and digestion with appropriate restriction enzymes, the flanking sequences were cloned into corresponding restriction sites in plasmid pMVp60, a plasmid containing VP60 gene and promoter P7.5 as described above.

A transfer vector was constructed as outlined in Fig. 1. First, recombination flank sequences were generated by PCR and cloned into plasmid pMvp60, which contains a VP60 gene and promotor P7.5. Those flanks directed the insertion of foreign DNA between MST3N gene, an intergenic site where can be knockout without interrupting the virus infection and replication [6]. In addition, P7.5-GFP cassette was placed into the recombination flank sequences to facilitate selection and identification of recombinant viruses.

A



B

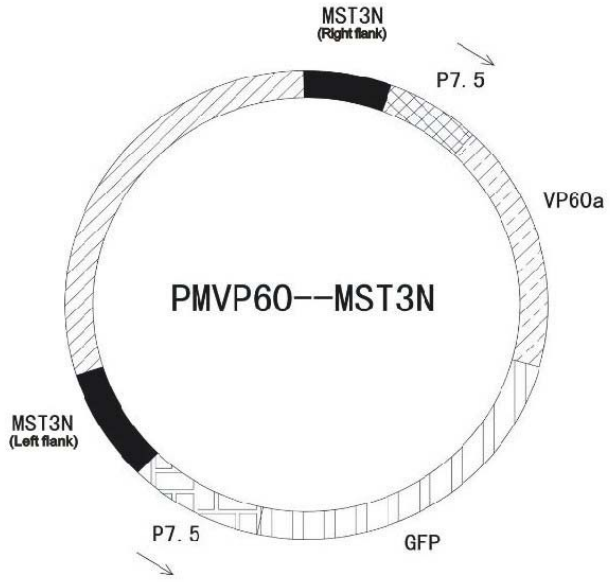


Fig 1 Schematic representation of MV genome and the transfer vector.(A) schematic representation of MV showing the inverted terminal repeats (ITR) and the right end position of the MST3N gene. Representation of MST3N gene sequences used as flanking sequences for homologous recombination. Positions of primers F1 and F2 used to amplify flank 1 and primers F3 and F4 used to amplify flank 2 were shown. (B) Scheme of the transfer vector used in the preparation of the recombinant myxoma viruses. GFP was transcripted in the leftward direction and VP60 is transcripted in the opposite direction .

C . Western immunoblotting

Monolayers of RK-13 cells were infected with MV BE4 strain or recombinant virus rBE4 at a multiplicity of infection of approximately 5 PFU per cell. When complete cytopathic effect was observed, the cells were harvested and the cell pellet was lysed with lysis buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1 mg of leupeptin per ml, 1 mMphenylmethylsulfonyl fluoride) for 20 min on ice. Subsequently, the lysate was centrifuged at 14,000 × g for 5 min. The proteins in the supernatants were separated by SDS-PAGE and transferred onto nitrocellulose membranes by electroblotting. The membranes were then blocked overnight at 4℃ with PBS+5% nonfat dry milk and incubated for 1 h at 37℃ either with a rabbit hyperimmune antiserum against RHDV for detecting VP60 protein or with antiserum of MV for detection of MV followed by FITC-labeled goat anti-rabbit IgG.

III. Results

A. Analysis of the MST3N gene

A novel myxoma virus early gene, MST3N, is a member of the eukaryotic sialyltransferase gene family positioned between genes MA51 and MA52 which located on 24 kb from the right end of the 163-kb myxoma virus genome [6]. The MST3N gene encodes a protein with significant amino acid similarity to a range of eukaryotic sialyltransferases with enzymatic activity toward the N and O-glycans of glycoproteins and oligosaccharide of glycolipids [7]. Inactivation of the MST3N gene in MV resulted in generation of the mildly attenuated virus establishing that the sialyltransferase is not required for infection or induction of clinical myxomatosis in genetically susceptible laboratory rabbits. In the absence of the sialyltransferase expression, disease symptoms are delayed [6]. Since the MST3N gene is not essential for the virus infection and replication, we could possibly use it as an insertion site.

B. Isolation of recombinant viruse MV-VP60

The RHDV capsid gene and the gene of GFP were placed separately under control of synthetic early promoter P7.5 and the recombinant virus generated by homologous recombination was selected by a combination of several rounds of limiting dilution and plaque selection under fluorescent microscope (data not shown). Recombination virus was further confirmed by PCR analysis using primers designed to amplify part of the VP60 gene (402bp) as shown in Fig. 2.

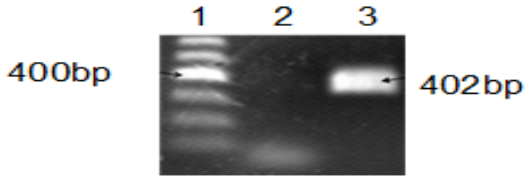


Fig. 2. PCR of the partialVP60 gene. 1. Marker; 2, Wt virus MV DNA template; 3, Recombinant virus rBE4 DNA template.

C. Expression of VP60 of recombinant MV-VP60.

Western blot analysis of RK-13cells infected with wt-BE4 or recombinant rBE4 showed the presence of specific polypeptides VP60 with the expected size in cell extracts from cultures infected with recombinant rBE4, but not in the wt-BE4 virus (Fig.3). A hyperimmune antiserum against RHDV recognized a protein with an apparent molecular mass of 60 kDa in cell lysates infected with recombinant BE4, which corresponded to the monomeric form of the VP60 capsid protein which is in agreement with the reports of others [8].

Fig . 3. Western Blot analysis of expression of VP60 in recombinant rBE4-infected RK-13 cells using a rabbit antiserum against the VP60 of RHDV. Line 1, prestained protein marker; line 2, cells infected with recombinant virus rBE4; line 3, cells infected with wt virus BE4.

IV. DISCUSSION

A number of vaccines are available to protect rabbits against myxomatosis and RHD. These vaccines have proven effective in the control of both diseases among domestic rabbits, but they are not suited to be used for wild rabbit vaccination [9]. Immunization of wildlife is difficult to achieve because such animals are free range, thus precluding the use of vaccines that require individual administration by conventional veterinary practices. With this in mind, we have explored the possibility of wild rabbit vaccination against both myxomatosis and RHD by using an MV-VP60 recombinant capable of spreading through rabbit populations by horizontal transmission .

This is the first report to use the GFP gene as a selection marker in poxvirus recombinant production. Previously, we tried to use the lacZ gene as a selection marker for recombinant virus. However, the length of lacZ gene fragment is too long , which might affect the efficiency of homologous recombination and stability of recombinant. And X-gal staining also increased the probability of false positive results to occur. To resolve this problem, we replaced the lacZ gene with the green fluorescent protein (GFP) gene, the expression of GFP under fluorescence microscope showing directly green fluorescence reduced greatly the chance of false positive. Furthermore, the length of the GFP gene about 800bp which is much shorter compared to that of the 3500 bp of lac Z gene improved dramatically the efficiency of homologous recombination and stability of recombinant .

Several facts led us to anticipate that the proposed transmissible vaccine could be useful for the control of myxomatosis and RHD among rabbit populations. First, recombinant poxvirus systems have been successfully developed as vectors for delivering a wide range of vaccine antigens to humans and animals. Viruses used include vaccinia virus[10], avipoxviruses, raccoonpoxvirus, capripoxvirus, swinepox virus and myxoma virus. Secondly, the expression of RHDV VP60 capsid protein in several heterologous systems has been shown to induce protective immunity. Remarkably, no indications of toxicity or side effects associated to the expression of VP60 have been reported [11]. In addition, molecular epidemiology studies have revealed a low genomic variation (less than 10% nucleotide divergence) within isolates collected from different geographic areas and over a period of several years [12].This result suggests that to date, a single RHDV serotype exists. Since this is also the case of MV, vaccination with a recombinant MV-VP60 is expected to provide effective protection against all currently circulating strains of MV and RHDV. Third, MV has some features that make it a good choice for the development of transmissible recombinant vaccines for rabbits, with respect to efficacy and safety.

Suitable insertion sites for heterologous genes have been described [13], and the ability of MV-based recombinants to induce a strong immune response, including mucosal immunity, has been established [14].

In this study, we choose the MV strain BE4 with mild virulence as the recombinant vaccine vector because the result of virulence test of BE4 is exactly what we expect : the injection sites do not show any scars, which means fleas will bite, then transmit the recombinant virus among wild rabbits. The further study would focus on the virulence of the recombinant MV BE4 in rabbits.

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