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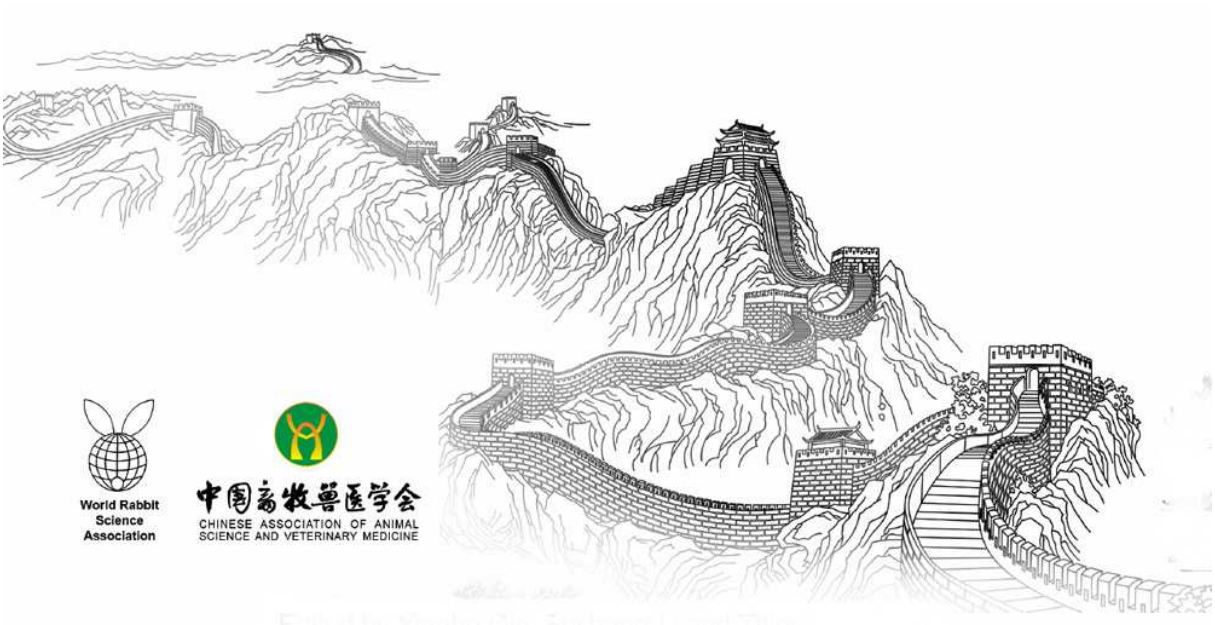

PRODUCTION, CHARACTERIZATION, AND EPITOPE MAPPING OF MONOCLONAL ANTIBODIES AGAINST DIFFERENT SUBTYPES OF RABBIT HEMORRHAGIC DISEASE VIRUS (RHDV)

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ABSTRACT

In 2010, a new rabbit hemorrhagic disease virus (RHDV) variant, designated RHDV2, was identified for the first time in Italy. Studies have shown that RHDV2 differs from RHDV1 (traditional RHDV) in terms of its antigenic profile and genetic characteristics. The VP60 protein of RHDV is a structural protein that plays important roles in viral replication, assembly, and immunogenicity. In this study, we immunized BALB/c mice with recombinant VP60 proteins from different RHDV subtypes. After three rounds of subcloning, type-specific positive hybridoma clones of RHDV1 and RHDV2 were further identified by an enzyme-linked immunosorbent assay, Western blotting, and an indirect immunofluorescence assay. Finally, three monoclonal antibodies (MAbs) (1D6, 1H2, and 3F2) that only recognize RHDV1, and four MAbs (1G2, 2C1, 3B7, and 5D6) that only recognize RHDV2 were identified. The epitopes recognized by these MAbs were mapped by Western blotting. Sequence analysis showed that the epitope sequences recognized by 1D6, 1H2, and 3F2 are highly conserved (98%) among RHDV1 strains, whereas the epitope sequences recognized by 1G2, 2C1, 3B7, and 5D6 are 100% conserved among RHDV2 strains. The high conservation of the epitope sequence showed that the screened MAbs were type-specific, and that they could distinguish different RHDV subtypes.

Keywords: Epitope, Rabbit hemorrhagic disease virus, Type-specific monoclonal antibody, VP60 protein

INTRODUCTION

Rabbit hemorrhagic disease virus (RHDV) is a member of the family *Caliciviridae*, genus *Lagovirus*, and it causes rabbit hemorrhagic disease (RHD), characterized by high morbidity and high mortality in both wild and domestic adult rabbits (Ferreira, 2006). Since first being reported in China in 1984, RHD spread worldwide within a few years. Infected adult rabbits succumb from 48 to 72 h post-infection, and death is often associated with hepatic, intestinal, and lymphoid necroses, as well as massive terminal intravascular coagulation. Like other caliciviruses, RHDV forms 28–32 nm diameter, non-enveloped, icosahedral virus particles that harbor a 7.4-kb positive-sense single-stranded RNA genome that encodes a 257 kDa polyprotein. The genome of RHDV has two open reading frames (ORFs): ORF1 encodes a polyprotein that is cleaved into non-structural components and the major structural protein, the capsid protein VP60, which is the main target of the host immune defense against RHDV and plays an important role in virus diagnosis and vaccine design; and ORF2 encodes the minor structural protein VP10 (Meyers,1991).

More recently, atypical RHD outbreaks in vaccinated rabbits, which have resulted in high mortality rates in young rabbits, have been changing the epidemiology of this disease, and they have coincided with the emergence and spread of a new RHDV variant. Various terms, such as “new variant”, “RHDVb” and “RHDV2”, have been used to describe the new RHDV variant, as a definitive RHDV nomenclature has not yet been agreed upon. This multiple naming of subclusters has caused confusion in the field, and for this study, we used the nomenclature of RHDV1 (traditional RHDV) and RHDV2 to define different subtypes of RHDV. The average nucleotide identity between RHDV2 and RHDV1-RHDVa is 82.4%, whereas the average amino acid similarity is about 89.2%. Studies have shown that RHDV2 differs from RHDV1 in terms of its antigenic profile and genetic characteristics, although partial cross-protection exists between RHDV1 and RHDV2, highlighting the need of using RHDV2-specific diagnostic assays to monitor the spread of this

new virus. To date, RHDV2 has been detected in Spain, France, Great Britain, and Italy (Le Gall-Recule,2011; Camarda,2014). At present, RHDV2 has not been reported in China. Therefore, it is very urgent to study the etiology, epidemiology, diagnosis, and control of RHDV.

In the present study, the VP60 proteins of RHDV1 and RHDV2 were used as immunogens to prepare RHDV type-specific MAbs by hybridoma fusion. Finally, three MAbs that specifically recognized RHDV1 and four MAbs that specifically recognized RHDV2 were identified. The epitopes recognized by these type-specific MAbs were also precisely mapped. Type-specific MAb preparation provides the foundation for the establishment of RHDV subtype-specific detection methods, and it has important significance for RHDV epidemiological investigations and phylogenetic analyses.

MATERIALS AND METHODS

Expression of recombinant VP60

The *vp60* genes of RHDV1 and RHDV2 were cloned into the vector pET-32a and then transformed into *E. coli* BL21 Star(DE3)pLysS cells to produce recombinant VP60 proteins named pVP60-1 and pVP60-2 used as immunogens after antigenicity testing by Western blotting. Rabbit hyperimmune serum against RHDV1 (generated in our laboratory) was used as the primary antibody for Western blotting as described below.

VP60 was also expressed in two eukaryotic expression systems. In the first system, a recombinant RHDV2 VP60 baculovirus was constructed, and the VP60 proteins of the different RHDV subtypes were expressed in insect cells using the Bac-to-Bac Baculovirus Expression System. sVP60-1 and sVP60-2 were used for indirect immunofluorescence assay (IFA) of the screened MAbs. In the second system, the *vp60* genes of RHDV1 and RHDV2 were cloned into the eukaryotic expression vector pcDN3.1(+)and expressed in HeLa cells.

Immunization protocol and hybridoma production

Adult female BALB/c mice, 6–8 weeks of age, were divided into two groups, and their hind leg muscles were injected intramuscularly with 100 μl of the purified pVP60s. Three days after the final ntravenous injection, serum antibody titers were monitored by ELISA, and high-titer antibody-producing animals were sacrificed for hybridoma production. Single splenocyte suspensions were prepared from the spleen of a selected mouse and fused with SP2/0 myeloma cells

Identification of MAbs targeting different RHDV subtypes

Based on a comparison of the RHDV1 and RHDV2 VP60 amino acid sequences, six short peptides, which had subtype-specific sequence , were synthesized (table 1). The RHDV TP strain (27 hemagglutination (HA) units), pVP60-1 (600 ng/well), pVP60-2 (600 ng/well), and the six short peptides (500 ng/well) were separately coated onto an ELISA plate and used as the detection antigens in an indirect ELISA with all of the positive hybridomas mentioned above. Based on the results of the indirect ELISA, the preliminary screening type-specific MAbs were further determined by Western blotting and IFA. Western blotting and IFA experiments were performed using Sf9 cells cultured in 96-well plates and HeLa cells cultured in 48-well plates.

Epitope mapping

The epitopes of type-specific MAbs were then defined by Western blotting. Linear epitopes of VP60 were predicted using the Immune Epitope Database (IEDB http://www.iedb.org/). Based on the predictions, six truncated polypeptides were designed. Recombinant truncated VP60 polypeptides were expressed and purified for Western blotting.

Table 1 Sequences of the synthesized polypeptides.

Name	Virus strain	Sequence	Length
R-A1	RHDV1	^{298}RRGSASYSGNNSTNVLQFWYAN^{319}	22 aa
R2-B1	RHDV2	^{298}DKGKASYPGSSSSNVLELWYAS^{319}	22 aa
R-A2	RHDV1	^{429}PNASAVTYTPQPDRIVTT^{446}	18 aa
R2-B2	RHDV2	^{429}PNSSAITYTPQPNRIVNA^{446}	18 aa
R-A3	RHDV1	^{405}YAVVTGTNQNPTG^{417}	13 aa
R2-B3	RHDV2	^{405}YGVATGINQATAG^{417}	13 aa

RESULTS AND DISCUSSION

To date, RHDV2 has not been detected in China. The non-enveloped RHDV virus capsid is constructed from the structural protein VP60, which is a major determinant of viral pathogenicity and antigenicity. The complete recombinant VP60 protein has been shown to be antigenic when expressed from different constructs, and to induce a detectable protective response (Perez-Filgueira,2007). VP60 is the main target for new vaccines, as well as for the development of immunity-based prophylactic, therapeutic, and diagnostic techniques for controlling RHDV. In this study, we produced seven RHDV type-specific MAbs and discovered four linear B-cell epitopes at the carboxyl-terminus of VP60.

The VP60-specific MAbs in this study were produced using recombinant VP60 proteins from different RHDV subtypes that were expressed in a prokaryotic expression system. Twenty of the most reactive MAbs against RHDV1 and 15 MAbs against RHDV2 were selected based on the ELISA results. Finally, three MAbs (1D6, 1H2, and 3F2) that only recognized RHDV1 were detected, while four MAbs (1G2, 2C1, 3B7, and 5D6) that only recognized RHDV2 were detected. The Western blotting and IFA results further validated the differences in epitope recognition of these MAbs. These seven MAbs were all IgG isotypes, and their antibody titers were quite high.

According to the Western blotting results, all seven MAbs positively reacted with denatured recombinant VP60, suggesting that their antigenic recognition sites were linear epitopes. To study their specificity in more detail and to map the epitopes bound by the MAbs, This identified the minimal sequence of the MAb 1D6-defined epitope as ^{256}RWNG***Q***^{260}, while MAbs 1H2 and 3F2 recognized the sequence ^{312}VL***QF***W^{316} (amino acids that differ between the RHDV1 and RHDV2 VP60 proteins are identified by italics), whereas MAb 2C1 recognized the linear antigen ^{324}***A***DNPIS^{329}, and MAbs 1G2, 3B7, and 5D6 recognized the sequence ^{294}***A***IDH***D***^{298}; the deletion of any residues from either end of these core sequences inhibited binding by the corresponding MAbs. It has been reported that one MAb 1G5, which recognizes a linear epitope with the sequence NPISQVAP (amino acids positions 326-333 of VP60 protein) located at loop L2 of P2 subdomain, and reacted with both RHDV1 and RHDV2 capsid proteins (Bárcen, 2015). Comparing the epitope of MAb1G5 with MAb 2C1 in our study, it showed that ^{324}A is the key amino acid that affect the epitope recognition ability of the MAb 2C1. Identification of epitopes showed that MAbs 1H2 and 3F2 recognized the same epitope ^{312}VLQFW^{316} and MAbs 1G2, 3B7 and 5D6 also recognized the same epitope ^{294}AIDHD^{298}. It was similar to MAbs 1H2 and 3F2 of RHDV1, MAbs 1G2, 3B7 and 5D6 of RHDV2 originated from different hybridoma culture wells, which means ^{312}VLQFW^{31} and ^{294}AIDHD^{298} might be immunodominant epitopes. The results of epitope mapping indicated that 1H2 and 3F2 belonged to one MAb, 1G2, 3B7 and 5D6 also belonged to one MAb as the same. Previous reports grouped RHDV epitopes into three different types based on Western blotting and/or ELISA reactivity: surface linear, surface conformational, and internal linear epitopes. The recognition abilities of these MAbs could indicate that they bind surface linear epitopes. The most exposed P2 sub-domain showed the highest degree of genetic variation, and it may contain an important epitope for anti-RHDV antibody production. However, the previously reported epitopes of RHDV were all located in the most amino-terminal or carboxyl-terminal regions of the capsid proteins (Kong, 2015). In this study, all seven MAb epitopes were located in the P2 sub-domain, except for that of MAb 1D6, which was located in the P1 sub-domain. To the best of our knowledge, this represents the first fine mapping of B-cell epitopes in the P2 sub-domain of VP60.

An analysis to assess the conservation of the identified MAb epitopes among members of the genus *Lagovirus* was performed. The results showed that the RHDV1 VP60 epitopes recognized by the MAbs are highly conserved (98%) among RHDV1 strains, whereas the RHDV2 VP60 epitopes recognized by the MAbs are completely conserved (100%) among RHDV2 strains. Furthermore, MAb 1D6 and MAbs 1H2 and 3F2 could also react with the RHDV1 TP strain and the RHDV1 Meiningen strain, which exhibited single amino acid differences according to the results of the sequence alignment. In conclusion, the ability of the screened MAbs to recognize different epitopes was quite stable, and they might be able to distinguish different subtypes of RHDV; thus, they are possibly type-specific MAbs. The key amino acid residues that account for the recognition differences of the MAbs are all completely conserved (Table 2).Previous results showed that RHDV2 differs from RHDV1 in terms of its disease duration, mortality rates, higher occurrence of subacute/chronic forms, and antigenic profile. Amino acids that affect the epitope recognition ability of the type-specific MAbs may also be the key residues that determine the antigenic differences between RHDV1 and RHDV2.

Table 2 Sequences analysis of the MAb antigenic epitopes of different RHDV subtypes.

Virus strain	Accession number	MAb 1D6	MAb 1H2/3F2	MAb 2C1	MAb 1G2/3B7/5D6
RHDV1	AAM21587.1	^{256}RWNGQ^{260}	^{312}VLQFW^{316}	I DNPIS	DIDHR
RHDV1	AAK85434.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	AAP15339.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	ABA46867.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	ABH10017.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	ACB28856.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	AEB26305.1	RWDGQ	ALQFW	I DNPIS	DIDHR
RHDV1	AEU09705.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	AEU09706.1	RWNGQ	VLQFW	VDNPIS	DIDHR
RHDV1	AEU09707.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	AEU09708.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	AFN69440.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	CAA75624.1	RWNVQ	VLQFW	VDNPIS	DIDHR
RHDV1	CAA80883.1	RWNGQ	VLQFW	I DNPIS	DIGHR
RHDV1	CAA75625.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	CAA75631.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	CAA75632.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	CAA75633.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	CAD91718.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	NP_740333.1	RWNGQ	VLQFW	I DNPIS	DIDHR
RHDV1	CAA60910.1	RWNGQ	VLQFW	VDNPIS	DIDHR
RHDVa	ABX38989.2	RWNGQ	VLQFW	I DNPIS	DIDHR
RCV	CAA65611.1	RWNCQ	VLQFW	VDNPIC	DIDHR
EBHSV	CAA66639.1	RWGAP	T I ETW	T TNPIS	DIDHR
RHDV2	CCH15347.1	RWNGE	VLELW	^{324}ADNPIS^{329}	^{294}AIDHD^{298}
RHDV2	CCH15344.1	RWNGE	VLELW	ADNPIS	AIDHD
RHDV2	CCH15345.1	RWNGE	VLELW	ADNPIS	AIDHD
RHDV2	CCH15346.1	RWNGE	VLELW	ADNPIS	AIDHD
RHDV2	CCH80663.1	RWNGE	VLELW	ADNPIS	AIDHD
RHDV2	CBZ39415.3	RWNGE	VLELW	ADNPIS	AIDHD
RHDV2	AGC11803.1	RWNGE	VLELW	ADNPIS	AIDHD
RHDV2	AGC11805.1	RWNGE	VLELW	ADNPIS	AIDHD
RHDV2	AGW27412.1	RWNGE	VLELW	ADNPIS	AIDHD
RHDV2	AGW27413.1	RWNGE	VLELW	ADNPIS	AIDHD
RHDV2	AGW27414.1	RWNGE	VLELW	ADNPIS	AIDHD
RHDV2	AHL89009.1	RWNGE	VLELW	ADNPIS	AIDHD

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Production, Characterization, and Epitope Mapping of Monoclonal Antibodies Against Different Subtypes of Rabbit Hemorrhagic Disease Virus (RHDV)

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The message

- RHDV2 ,a new rabbit hemorrhagic disease virus (RHDV) variant, has spread in Europe.
- To date, RHDV2 has not been detected in China.
- Type-specific MAb preparation provides the foundation for detection methods, epidemiological investigations and phylogenetic analyses.

Methods

1 Expression of recombinant VP60 in prokaryotic expression system and eukaryotic expressing system

2 Production of MAbs against recombinant VP60 proteins

3 Identification and characterization of RHDV type-specific Mabs and their linear B-cell epitopes.

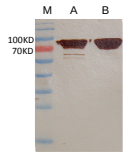
Introdution

We immunized BALB/c mice with recombinant VP60 proteins from different RHDV subtypes. Ttype-specific positive hybridoma were further identified by immunological method . Mabs (1D6, 1H2, and 3F2) that only recognize RHDV1, and MAbs (1G2, 2C1, 3B7, and 5D6) that only recognize RHDV2 were identified. The epitopes sequence recognized by these MAbs were highly conserved ,which showed that these MAbs were type-specific, and could distinguish different RHDV subtypes.

Results

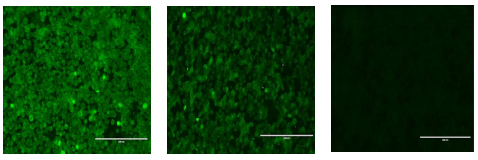
- Expression of recombinant VP60 in prokaryotic expression system and eukaryotic expressing system

Expression of recombinant VP60 in *Ecoli*.BL21(DE3)plys



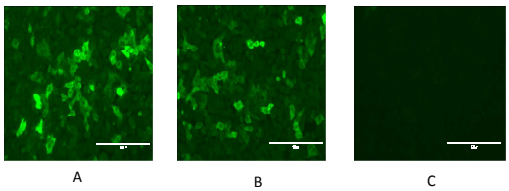
M:Protein Marker；
1:pET-R-VP60；2: pET-R2-VP60

Expression of recombinant VP60 in Hella cell



A:pcDNA-R1-VP60/Hela；B:pcDNA-R2-VP60/Hela；
C:pcDNA3.1(+) /Hela；

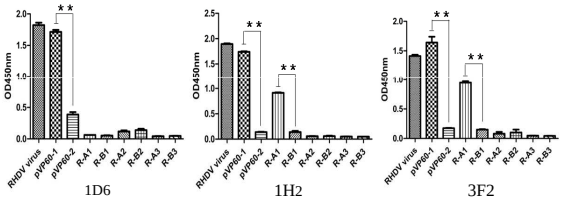
Expression of recombinant VP60 in SF9 cell

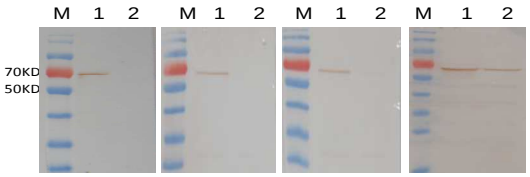


A：Bac-R1-VP60 recombinant baculovirus /Sf9；B：Bac-R2-VP60 recombinant baculovirus /Sf9；C：Baculovirus/ Sf9

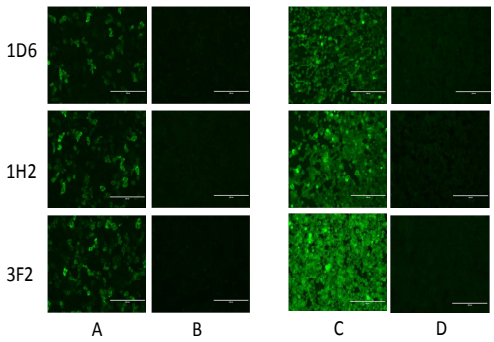
- Identification and characterization of RHDV type-specific Mabs and their linear B-cell epitopes

Specific McAbs against RHDV1





M: Protein Marker；1: pcDNA-R1-VP60/Hela；2: pcDNA-R-VP60/Hela

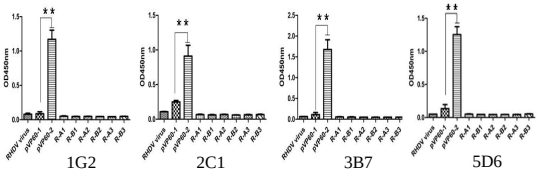


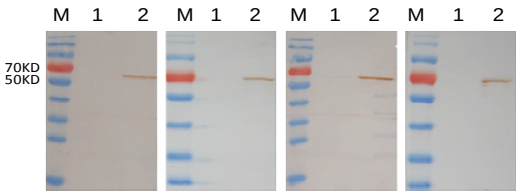
A：pcDNA-R1-VP60/Hela；B：pcDNA-R2-VP60/Hela；
C：Bac-R1-VP60 recombinant baculovirus /Sf9；
D：Bac-R2-VP60 recombinant baculovirus /Sf9

Virus strain	Accession number	MAb 1D6	MAb 1H2/3F2
RHDV1	AAM21587.1	259RWNGQ-260Q	312VLQFW316
RHDV1	AAK85434.1	RWNGQ	VLQFW
RHDV1	AAP15339.1	RWNGQ	VLQFW
RHDV1	ABA16867.1	RWNGQ	VLQFW
RHDV1	ABH10017.1	RWNGQ	VLQFW
RHDV1	ACB28856.1	RWNGQ	VLQFW
RHDV1	AEB26305.1	RWNGQ	ALQFW
RHDV1	AEU09705.1	RWNGQ	VLQFW
RHDV1	AEU09706.1	RWNGQ	VLQFW
RHDV1	AEU09707.1	RWNGQ	VLQFW
RHDV1	AEU09708.1	RWNGQ	VLQFW
RHDV1	AFN69440.1	RWNGQ	VLQFW
RHDV1	CAA75624.1	RWNVQ	VLQFW
RHDV1	CAA80883.1	RWNGQ	VLQFW
RHDV1	CAA75625.1	RWNGQ	VLQFW
RHDV1	CAA75631.1	RWNGQ	VLQFW
RHDV1	CAA75632.1	RWNGQ	VLQFW
RHDV1	CAA75633.1	RWNGQ	VLQFW
RHDV1	CAD91718.1	RWNGQ	VLQFW
RHDV1	NP_740333.1	RWNGQ	VLQFW
RHDV1	CAA60910.1	RWNGQ	VLQFW
RHDVa	ABX38989.2	RWNGQ	VLQFW
RCV	CAA65611.1	RWNCQ	VLQFW
EBHSV	CAA66639.1	RWGAP	TTETW
RHDV2	CCH15347.1	RWNGE	VLELW
RHDV2	CCH15344.1	RWNGE	VLELW
RHDV2	CCH15345.1	RWNGE	VLELW
RHDV2	CCH15346.1	RWNGE	VLELW
RHDV2	CCH80663.1	RWNGE	VLELW
RHDV2	CBZ39415.3	RWNGE	VLELW
RHDV2	AGC11803.1	RWNGE	VLELW
RHDV2	AGC11805.1	RWNGE	VLELW
RHDV2	AGW27412.1	RWNGE	VLELW
RHDV2	AGW27413.1	RWNGE	VLELW
RHDV2	AGW27414.1	RWNGE	VLELW
RHDV2	AHL89009.1	RWNGE	VLELW

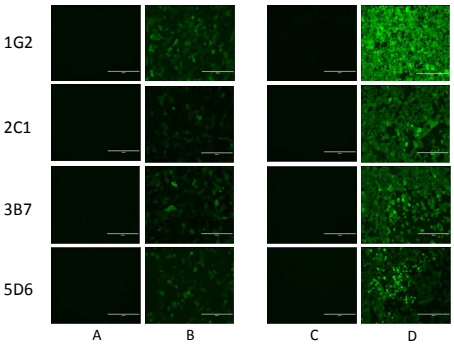
RHDV1 VP60 epitopes that are recognized by Mabs(1D6/1H2/3F2) are highly conserved (98%)

Specific McAbs against RHDV2





M: Protein Marker；
1: pcDNA-R1-VP60/Hela；2: pcDNA-R-VP60/Hela



A：pcDNA-R1-VP60/Hela；B：pcDNA-R2-VP60/Hela；
C：Bac-R1-VP60 recombinant baculovirus /Sf9；
D：Bac-R2-VP60 recombinant baculovirus /Sf9

Virus strain	Accession number	MAb 2C1	MAb 1G2/3B7/5D6
RHDV1	AAM21587.1	IDNPIS	DIDHR
RHDV1	AAK85434.1	IDNPIS	DIDHR
RHDV1	AAP15339.1	IDNPIS	DIDHR
RHDV1	ABA46867.1	IDNPIS	DIDHR
RHDV1	ABH10017.1	IDNPIS	DIDHR
RHDV1	ACB28856.1	IDNPIS	DIDHR
RHDV1	AEB26305.1	IDNPIS	DIDHR
RHDV1	AEU09705.1	IDNPIS	DIDHR
RHDV1	AEU09706.1	VDNPIS	DIDHR
RHDV1	AEU09707.1	IDNPIS	DIDHR
RHDV1	AEU09708.1	IDNPIS	DIDHR
RHDV1	AFN69440.1	IDNPIS	DIDHR
RHDV1	CAA75624.1	VDNPIS	DIDHR
RHDV1	CAA80883.1	IDNPIS	DIGHR
RHDV1	CAA75625.1	IDNPIS	DIDHR
RHDV1	CAA75631.1	IDNPIS	DIDHR
RHDV1	CAA75632.1	IDNPIS	DIDHR
RHDV1	CAA75633.1	IDNPIS	DIDHR
RHDV1	CAD91718.1	IDNPIS	DIDHR
RHDV1	NP_740333.1	IDNPIS	DIDHR
RHDV1	CAA60910.1	VDNPIS	DIDHR
RHDVa	ABX38989.2	IDNPIS	DIDHR
RCV	CAA65611.1	VDNPIC	DIDHR
EBHSV	CAA66639.1	TTNPIS	DIDHR
RHDV2	CCH15347.1	324ADNPIS329	294AIDHD298
RHDV2	CCH15344.1	ADNPIS	AIDHD
RHDV2	CCH15345.1	ADNPIS	AIDHD
RHDV2	CCH15346.1	ADNPIS	AIDHD
RHDV2	CCH80663.1	ADNPIS	AIDHD
RHDV2	CBZ39415.3	ADNPIS	AIDHD
RHDV2	AGC11803.1	ADNPIS	AIDHD
RHDV2	AGC11805.1	ADNPIS	AIDHD
RHDV2	AGW27412.1	ADNPIS	AIDHD
RHDV2	AGW27413.1	ADNPIS	AIDHD
RHDV2	AGW27414.1	ADNPIS	AIDHD
RHDV2	AHL89009.1	ADNPIS	AIDHD

RHDV2 VP60 epitopes recognized by MAbs (1G2/2C1/3B7/5D6)are completely conserved (100%)

Conclusion

Type-specific MAb preparation provides the foundation for the establishment of RHDV subtype-specific detection methods, and it has important significance for RHDV epidemiological investigations and phylogenetic analyses.