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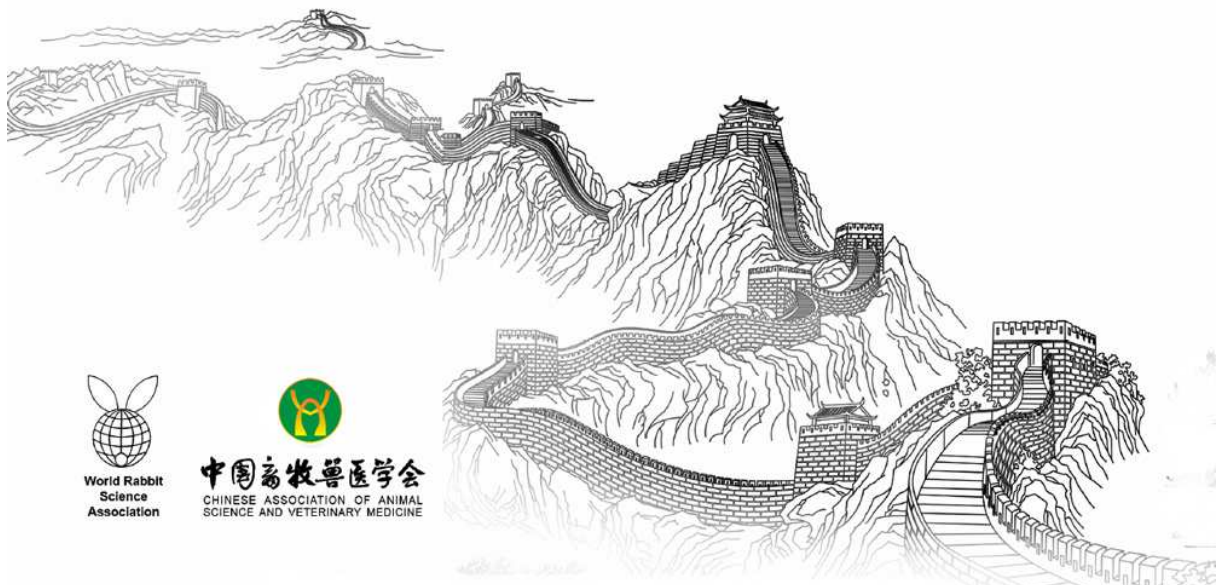
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## A NEW VARIANT OF RABBIT HEMORRHAGIC DISEASE VIRUS G2 STRAIN ISOLATED IN CHINA

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### ABSTRACT

To investigate the genetic variability and evolution of rabbit hemorrhagic disease virus (RHDV) strains in China, VP60 gene sequences of eight new isolates collected from farms with RHD occurrences in China between 2009 and 2014 were analyzed, and compared with the reference sequence of the vaccine strain WF/China/2007. We conducted a comprehensive analysis of the Chinese RHDV strains, including hemagglutination tests, western blot and phylogenetic analysis, and identified a new distinct antigenic variant. Specifically, strain HB/2014 collected in North China was identified as a non-hemagglutinating strain, and belongs to the original RHDV (G1–G5) group. The other seven isolates were classified in genogroup G6 (RHDVa), which was widely distributed across China before 2014. Collectively, these data provide new tools and insight for further understanding the molecular evolution of RHDV in China.

**Key words:** RHDV, VP60, Non-hemagglutinating, Phylogenetic analysis, China

### INTRODUCTION

Rabbit hemorrhagic disease (RHD) is an acute fatal infectious disease first described in 1984 (Liu et al., 1984), which has been reported worldwide (Forrester et al., 2006), leading to epidemics with high morbidity and mortality (Alonso et al., 1998; Hukowska-Szematowicz et al., 2012; Tunon et al., 2003).

Rabbit hemorrhagic disease virus (RHDV) is a positive-sense, single-stranded RNA virus of approximately 7 k nucleotides with two open reading frames (ORFs) (Meyers et al., 1991; Wirblich et al., 1996). The 3' terminus of ORF1 encodes the viral coat protein VP60 which is subdivided into three domains: NTA, S domain, and P domain (Wang et al., 2012). RHDV strains can be classified, based on their ability to agglutinate red blood cells, in non-hemagglutinating and hemagglutinating viruses. On the basis of the VP60 nucleotide sequences, RHDV strains have been divided into six genogroups (G1–G6) (Le Gall-Recule et al., 2003); genogroup G6 corresponds to the first antigenic variant of RHDV and has been designated as RHDVa (Capucci et al., 1998). Recently, a new RHDV-related virus, with the proposed name RHDV2, was found in France (Le Gall-Recule et al., 2013; Puggioni et al., 2013), the Iberian peninsula (Abrantes et al., 2013; Dalton et al., 2012), and Scotland (Baily et al., 2014) and Italy (Puggioni et al., 2013; Camarda et al., 2014). According to its unique genetic and antigenic profile, RHDV2 is being considered a newly emerged virus and not a variant of RHDV (Le Gall-Recule et al., 2013). However, the specific RHDV strains responsible for recent outbreaks in China have not yet been reported. In this study, we aimed to understand the molecular characterization of the recently collected RHDV strains and further describe the genetic profile of the new HA-negative RHDV variant in China.

### MATERIALS AND METHODS

#### Virus samples

Eight new RHDV isolates collected from rural farms experiencing RHDV outbreaks in six different provinces in China between 2009 and 2014 were as follows: HB/2014 (Hebei Province, Accession Number: KU207100), DQ/China/2010 (Heilongjiang Province, Accession Number: KJ814618), CC/China/2011 (Jilin Province,

Accession Number: KJ814617), LW/China/2011 (Shandong Province, Accession Number: KJ814621), NJ-2009/China (Jiangsu Province, Accession Number: HM623309), LH/China/2011 (Jiangsu Province, Accession Number: KJ814620), LY/China/2010 (Jiangsu Province, Accession Number: KJ814622), and JA/China/2011 (Jiangxi Province, Accession Number: KJ814619). These strains were compared to the reference strain WF/China/2007 (Accession Number: FJ794180), which has been developed as a commercial inactivated vaccine strain, widely used in China (Nanjing Tianbang Bio-Industry Co., Ltd.).

### Hemagglutination assay (HA)

The strains were subjected to hemagglutination test. Liver samples were homogenized (20% in PBS), and frozen and thawed at least twice. The hemagglutination test was carried out in U-shaped microtiter plates containing 50  $\mu$ L of PBS (pH 6.5). Fifty-microliter suspensions of homogenized liver samples were two-fold serially diluted and placed in U-shaped plates, and then further incubated with an equal volume of 1% human O erythrocytes at 4°C, 25°C, or 37°C, respectively, and hemagglutination was visually determined 30 min later. The highest dilution of each sample causing complete agglutination of red cells was considered the end point of the reaction.

### Phylogenetic analysis

For a more detailed analysis of the collected RHD samples and to understand the nature of RHDV epidemics in China, we compared VP60 gene sequences among the strains. The VP60 genes (nt 5191–7144) were amplified by using a pair of specific primers designed by our team (sense, 5'-CGGCAACAAGCATACACTC-3' and antisense 5'-CAACCCATTCTCCAAAGC-3'), and sequenced. We conducted a phylogenetic analysis of 109 VP60 gene sequences using MEGA 5.2 with the maximum likelihood (ML) approach based on the Kimura 2-parameter model. Reliability of the nodes was assessed with a bootstrap resampling procedure consisting of 1000 replicates.

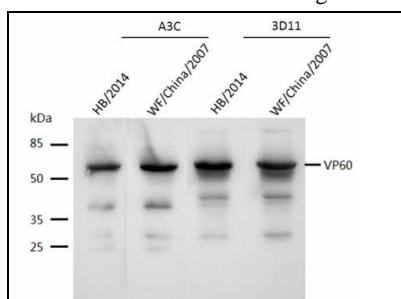
## RESULTS AND DISCUSSION

The results of the hemagglutination tests showed that the vaccine strain WF/China/2007 showed partial hemagglutination, while the RHDV strain HB/2014 failed to hemagglutinate at 4°C, 25°C, and 37°C (Table 1). The other seven strains also showed only partial hemagglutination, similarly to WF/China/2007, with negative reactions at 25°C and 37°C. This result indicated that HB/2014 is a non-hemagglutination strain. Western blotting assay was used to verify the integrity of the main capsid protein (VP60) of HB/2014 compared to WF/China/2007 with monoclonal antibodies (MAbs) specific for the S-domain (A3C) and P-domain (3D11). The results showed no degradation of the capsid protein (Figure 1), indicating that the lack of hemagglutination capacity of HB/2014 might instead be due to mutation of amino acids in the particle surface domain of VP60.

**Table 1:** Hemagglutination titers of the HB/2014 strain compared with the reference virus WF/China/2007 at different temperatures.

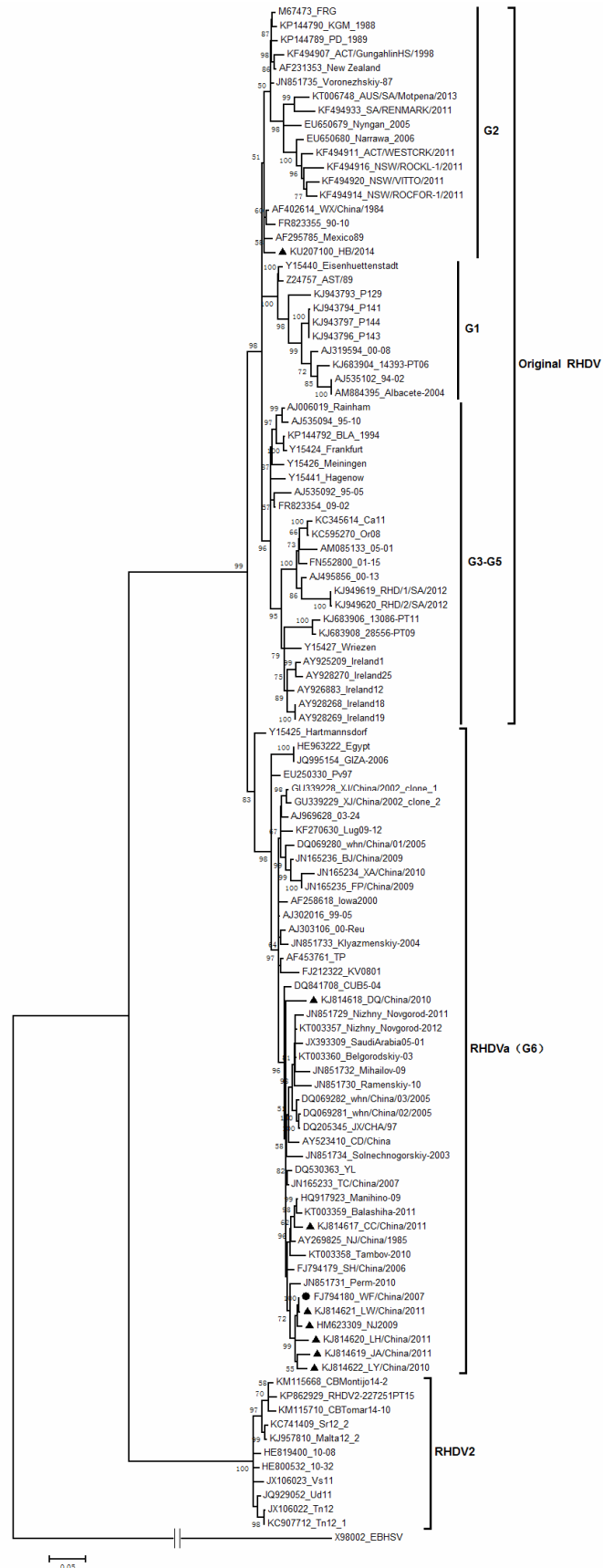
RHDV strains	4°C (pH 6.5) <sup>a</sup>	25°C (pH 6.5) <sup>a</sup>	37°C (pH 6.5) <sup>a</sup>
HB/2014	<2	Negative	Negative
WF/China/2007	1024	<2	Negative
SPF liver	Negative	Negative	Negative

<sup>a</sup> HA titer was tested for homogenized suspensions of liver samples with a dilution of 1:5 ratio by PBS (pH 6.5).



**Figure 1:** Immunoblot of the capsid protein from the HB/2014 strain and the reference strain WF/China/2007, probed with MAbs A3C and 3D11. Molecular weights are indicated.

The amplified VP60 genes were sequenced and the phylogenetic analysis was performed. Seven of the eight collected strains clustered in the genogroup 6 (RHDVa), which contained all Chinese strains except for WX/China/1984 isolated before 2014. Interestingly, the HB/2014 strain collected from North China in 2014 was a G2 isolate, and the only isolate belonging to the original RHDV cluster (G1–G5) among those collected after 1984 (Figure 2), indicating that RHDVa strains have not completely replaced the original RHDV strains in China. The undiscovered nature of G2 isolates might be attributed to their non-hemagglutinating characteristic, making them easy to ignore in clinical detection. Our results suggest that the G2 and G6 genogroups represent two different evolutionary directions of RHDV in China.



**Figure 2:** Maximum-likelihood phylogenetic tree of 109 nucleotide sequences of the rabbit hemorrhagic disease virus (RHDV) capsid protein (VP60) gene.

## CONCLUSIONS

In summary, analysis of the new non-hemagglutinating RHDV isolate HB/2014 suggests the possibility that the G2 group of RHDV has not yet disappeared in China. Furthermore, the results of phylogenetic analysis highlight the diversity of the collected RHDV strains, which can be assigned into different genogroups. These results should serve as a useful baseline to help further uncover the nature of epidemics and the molecular evolution of RHDV in China.

## ACKNOWLEDGEMENTS

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