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Brief Report

Evidence for recombination in the major capsid gene VP60 of the rabbit haemorrhagic disease virus (RHDV)

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Summary

Rabbit haemorrhagic disease (RHD) is a highly fatal disease caused by a virus of the family *Caliciviridae*. Whereas recombination is well documented in other members of this family, the extent of recombination has so far not been studied in RHDV. To reach a better evaluation of the possible role of recombination in the evolution of RHDV virulence, we have searched for recombination events in RHDV by analysing 43 complete sequences of the major capsid gene VP60. Phylogenetic analyses revealed two well separated groups. Clear evidence for recombination was found for the Hartmannsdorf strain which shows different phylogenetic profiles depending on the region of the capsid examined.

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The disease is highly contagious and is characterised by high mortality rates and high morbidity in both wild and domestic rabbits (*Oryctolagus cuniculus*). Since the first outbreak in China in 1984, RHD rapidly became endemic in several countries with consequent economic losses. The aetiological agent, rabbit haemorrhagic disease virus (RHDV), is a positive-sense, single-stranded RNA virus. Of the two open reading frames (ORFs), ORF1 encodes a polyprotein which is cleaved into non-structural components and the major structural protein, i.e., the capsid VP60 [28, 32]. RHDV belongs to the genus *Lagovirus* of the family *Caliciviridae* [32]. This family includes human pathogens, such as Norwalk virus, as well as other animal pathogens, *e.g.* feline calicivirus (FCV), vesicular exanthema virus of swine (VEV), San Miguel sea lion virus (SMSV) and European brown hare syndrome virus (EBHSV) [16, 33].

Phylogenetic analyses of the RHDV strains collected worldwide showed that they cluster into several genogroups which were found to be more correlated with the year of isolation than to the geographic location [21, 22, 25, 30]. Phylogenetic studies done so far were based upon the gene of the major capsid protein VP60 of RHDV. The VP60 protein is usually divided into six regions (A–F), according to the nomenclature proposed by Neill

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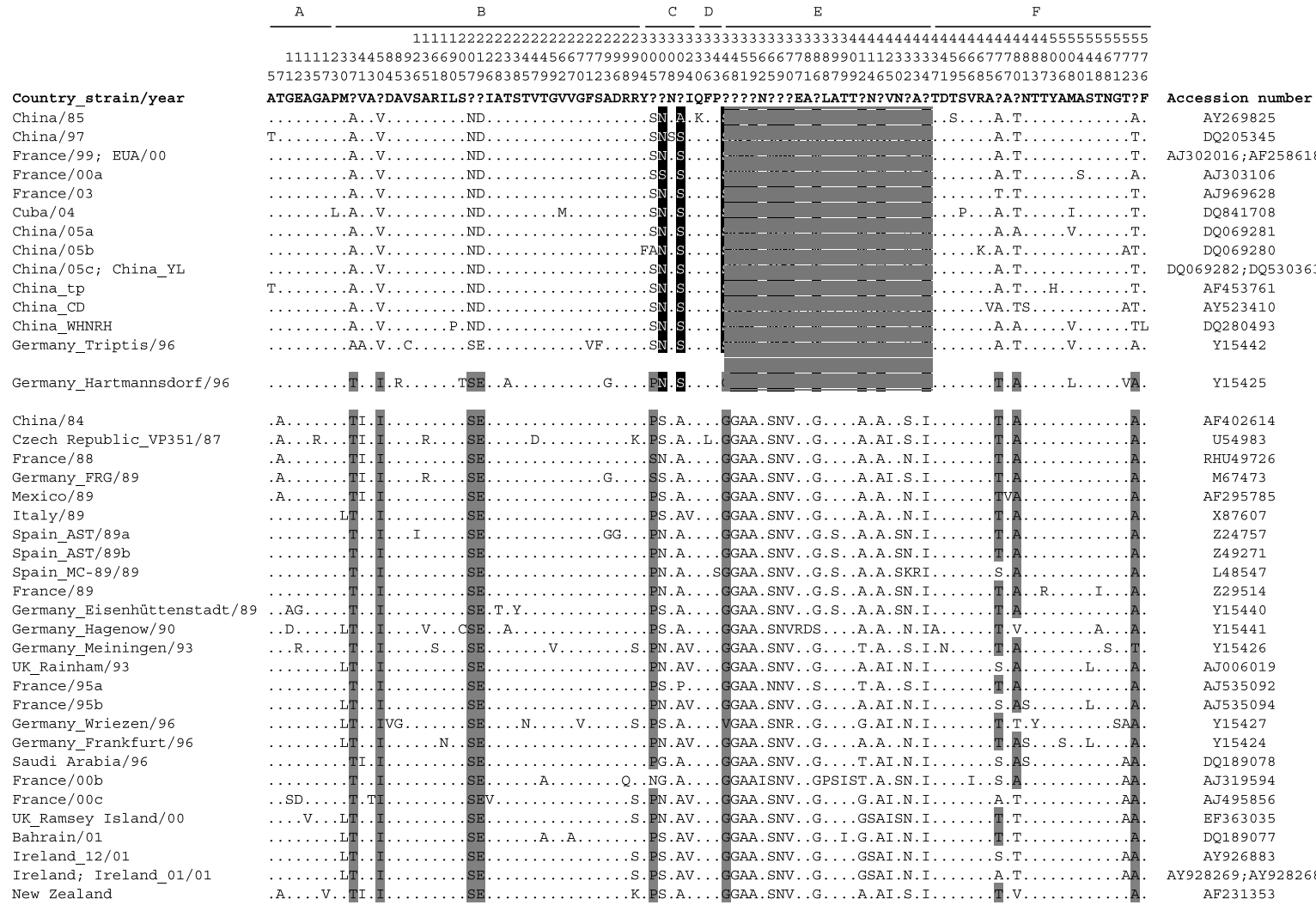
Table 1. Amino acid polymorphism found in the 43 VP60 sequences



Amino acid polymorphic positions and the different regions are indicated at the *top*. The characteristic amino acids shared between the putative recombinant strain and group I or group II are shaded in light *grey* or *black*, respectively. *Dots* represent identity with the consensus sequence (constructed with the 43 VP60 sequences; question marks represent ambiguous amino acids). The shaded area highlights the similarity between the putative recombinant strain and group II in the region E commonly used in phylogenetic studies. The year of isolation was only included when available.

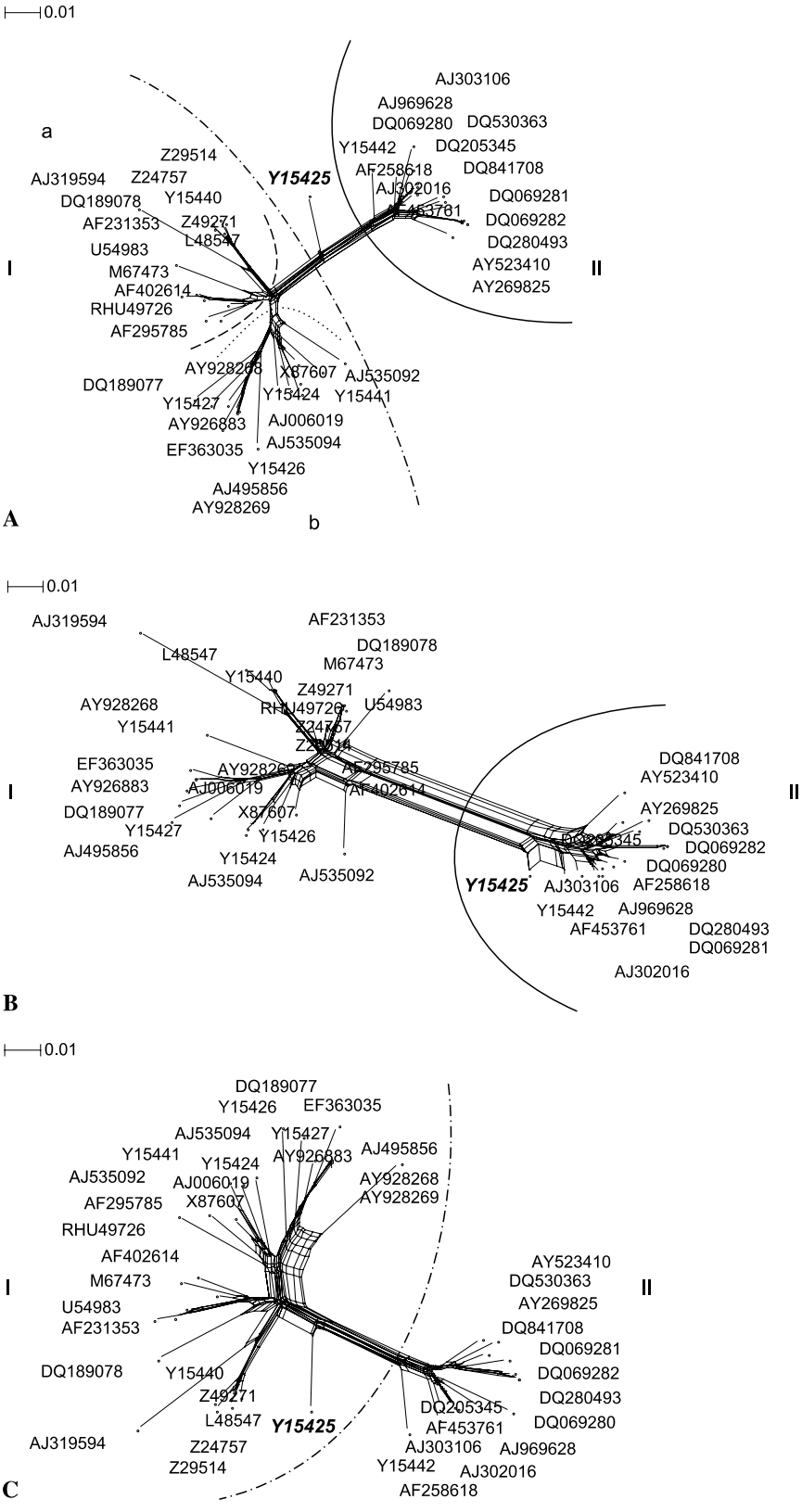


Fig. 1. Split decomposition analyses of the 43 VP60 nucleotide sequences (**A**) including the defined A–F regions, (**B**) including only hypervariable region E, (**C**) excluding nucleotide positions 1030–1302, which correspond to region E

[29]. In most species of caliciviruses, the C-terminal half of this protein is the most variable [36]. Cryo-electron microscopy studies and a more recent modelling study have revealed that the capsid is composed of two concentric shells: the inner shell made by the N-terminal half of the VP60 protein (S domain), which protects and contains the genome, and the outer shell composed of the variable C-terminal half of the protein (P domain), which contains the two highly variable regions, C and E, and the main antigenic region [3, 7, 8, 26, 28].

Although most genetic diversity in RNA viruses occurs during RNA replication by point mutations caused by RNA-dependent polymerases with no proofreading activity, it appears that recombination, rather than point mutations, has the most profound impact (reviewed in Ref. [20]). Thus, understanding recombination can be helpful in unravelling the evolution of pathogens and drug resistance. In contrast to other members of the family *Caliciviridae*, where recombination is a well documented (e.g. [9, 16, 34]), in RHDV its occurrence has only been suggested [12]. In this study, we present evidence for a recombination event in RHDV.

All of the complete sequences of the major capsid gene VP60 of RHDV available in GenBank were retrieved and aligned using the BioEdit software version 7.0.5.3 [15] (the accession numbers, location and year of isolation of the 43 sequences used in this study are listed in Table 1). The most common tree-building methods implicitly assume that branches never interact, which is not appropriate for analysing recombination events that produce networks of branches. The SplitsTree program [17] allows for these situations, making it a suitable method for the representation of conflicting phylogenetic signals [2]. The split decomposition analyses of the VP60 gene were constructed according to the *Neighbour-Net* method [4] and are shown in Fig. 1. A first analysis using the complete VP60 sequence (Fig. 1A) produced two distant groups (I and II), one of which can be subdivided into two well-supported subgroups (a, b). Only one sequence, the Hartmannsdorf strain (GenBank accession number Y15425), could not be assigned to one of these groups. Then, analyses were performed by using either the hypervariable region E (setting E, which is commonly used in phylogenetic studies) or by using the remaining regions (setting non-E, i.e. regions A–D and F). In both settings, the Hartmannsdorf strain could now be assigned to one or the other group, which, however, differed according to the setting. Thus, whereas sequence Y15425 clusters with group II in the E setting (Fig. 1B), it clusters with group I in non-setting E (Fig. 1C). This observation strongly supports a chimeric origin of this strain, most likely as the outcome of genome recombination. However, it should be mentioned that the Hartmannsdorf strain does not perfectly cluster with the group I viruses in the

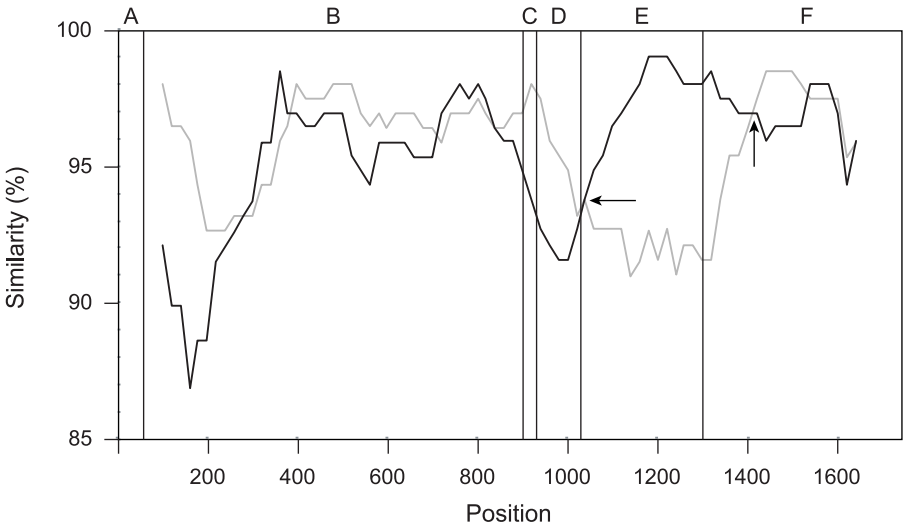


Fig. 2. Nucleotide identity plot for the Hartmannsdorf strain when compared to all the sequences composing the groups I (*grey*) and II (*black*) defined by the Split decomposition analyses. The *bars* define the different regions (indicated at the *top*). The *arrows* indicate the inferred recombination *breakpoints*. *Window* 200 bp; *Step* 20 bp, *Gapstrip* On, Kimura (2-parameter), T/t 2.0

non-E regions. This could indicate that one of the parental viruses might be a so-far unknown strain related to the group I viruses.

Following the methods applied in previous related studies (e.g. [16]), we used the SimPlot software [23] to locate the potential recombination site in the putative ''recombinant'' strain, by conducting nucleotide pairwise sequence comparisons between this strain and all of the sequences composing groups I and II, respectively (see Fig. 1A). In this analysis, the putative recombinant strain is compared to the sequences composing the two putative parental groups that gave origin to it. Therefore, the recombinant is more similar to one of the parental groups than to the other up to the site where recombination occurs. Then there will be a marked decrease in similarity between the recombinant and this parental group and an increase in similarity with the other parental group. The similarity plot in Fig. 2 shows in the beginning of region B a decrease in similarity between the ''recombinant'' strain and the viruses composing both groups I and II. While there is a marked drop in the overall nucleotide similarity in regions C and D, in region E, the ''recombinant'' strain is clearly more similar to the members of group II (identity similarity between 94 and 99%) than to those of the other group (identity similarity between 91 and 94%). In region F, however, it becomes more similar to the members of group I. The analysis indicates a potential recombination site localized at the beginning of region E and around nucleotide position 1400 (region F). The multiple crossings of the lines in the similarity plot could be explained by the absence of the true parental virus from group I. Studies on other caliciviruses have localised a consistent recombination site between the polymerase and capsid genes [5, 9, 18, 19, 31], upstream of region E, while recombination within the capsid gene has already been reported for the genus *Norovirus* [34].

At the amino acid level (Table 1) in the VP60 protein, we recognised only two groups (coincident with the groups defined by the SplitsTree analyses). The ''mixed'' origin of the Hartmannsdorf strain is confirmed by the sharing of amino acids motifs that are characteristic for each group (Table 1, highlighted), in agreement with the similarity plot. Again, in region E, the Hartmannsdorf strain has more sequence similarity to group II, in contrast to what is observed at the other regions. Our knowledge of the true RHDV phylogeny might have been limited by the fact that most of the phylogenetic studies on RHDV only focus on region E [1, 8, 10–14, 22, 24, 25, 27, 30], masking situations like the one reported here.

The fact that the Hartmannsdorf strain is represented by one single sequence might raise some questions about its origin. If a mixed population of viruses was present in the original sample, the possibility of a recombination event can not be excluded. Indeed, this strain was isolated from a rabbit that had previously been vaccinated [35]. The commercial inactivated vaccine used, RIKA-VACC (Riemseir Arzneitmittel AG, Greifswald Riems, Germany), is made from liver homogenates of rabbits infected with the RHDV strain Eisenhüttenstadt (GenBank accession number Y15440). However, the Hartmannsdorf strain has several characteristic amino acids that are not shared with the Eisenhüttenstadt strain, ruling out the possibility of a virus-vaccine recombination. Additionally, as several recombination events have been reported for members of the family *Caliciviridae* (e.g. [9, 16, 34]), it would, *a posteriori*, not be surprising that such recombinations do also occur in members of the genus *Lagovirus*. Recombination can be responsible for loss of efficacy of the vaccines so far developed and may contribute to persistent RHDV infections. For example, in AIDS, the numerous circulating recombinant forms of the human immunodeficiency virus (HIV) are one of the major obstacles to controlling its spread (reviewed in Ref. [6]).

In conclusion, gene exchange within the lagovirus genogroups might be more frequent than currently perceived. As this can have serious implications for disease diagnosis and control of RHDV by the host immune response, further research is needed for evaluating the extent of recombination among members of the genus *Lagovirus*.

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