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Phylogenetic analysis of rabbit haemorrhagic disease virus in France between 1993 and 2000, and the characterisation of RHDV antigenic variants

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Summary. The first molecular epidemiological study of *Rabbit haemorrhagic disease virus* undertaken in France between 1988 and 1995, identified three genogroups, two of which (G1, G2) disappeared quickly. We used immunocapture-RT-PCR and sequencing to analyse 104 new RHDV isolates collected between 1993 and 2000. One isolate was obtained in 2000 from a French overseas territory, the Reunion Island. The nucleotide sequences of these isolates were aligned with those of some French RHDV isolates representative of the three genogroups previously identified, of some reference strains and German and American RHDV antigenic variants. Despite the low degree of nucleotide sequence variation, three new genogroups (G4 to G6) were identified with significant bootstrap values. Two of these genogroups (G4 and G5) were related to the year in which the RHDV isolates were collected. Genogroup G4 emerged from genogroup G3, which has now disappeared. Genogroup G5 is a new independent group. The genogroup G6 contained an isolate collected in mainland France in 1999 and the isolate collected from the Reunion Island, as well as German and American RHDV variants. Multiple sequence alignments of the VP60 gene and antigenic analysis with monoclonal antibodies demonstrated that these French isolates are two new isolates of the RHDV variant.

Introduction

Rabbit haemorrhagic disease (RHD) is a highly contagious and fatal disease that affects domesticated and wild rabbits of the genus *Oryctolagus cuniculus*. RHD has a high mortality rate and is responsible for large economic losses to rabbit producers. The disease was first described in China in 1984 [16] and subsequently spread to Europe. The RHD virus (RHDV) is currently endemic in most parts of the world. Morphological, biochemical, protein and genomic studies have shown that the virus is a calicivirus, an icosahedral non-enveloped positive-sense single-stranded RNA virus with a diameter of about 35 nm [22, 23, 25, 17, 24]. Until now, the attempts at *in vitro* propagation of RHDV failed. RHDV was recently designated as the type species of the genus *Lagovirus* [11], which includes the *European brown hare syndrome virus* (EBHSV), a highly pathogenic virus of hares (European brown hare *Lepus europaeus* and "varying hares" *L. timidus*). In 1996, a non-pathogenic virus preliminarily called Rabbit calicivirus (RCV), that is more closely related to RHDV than to EBHSV was described [6].

Phylogenetic studies have evaluated the genetic variation between RHDV isolates collected over a period of several years. It is of particular interest to monitor the molecular phylogeny of RHDV over time as a model for the appearance of disease and isolates. Although RHDV is a RNA virus, the RNA and protein sequences of different isolates of RHDV are highly conserved [18, 21, 14, 1]. However, despite the low level of genetic variation, well-defined genogroups have been identified in the phylogenetic trees established by Nowotny et al. [21] and by Le Gall et al. [14]. These studies, carried out with 39 isolates collected between 1987 and 1995 from 17 countries and with 56 French isolates collected over the same period, respectively, suggested that the distribution of the isolates within the different genogroups is related to the year in which they were collected. Antigenic studies did not find any antigenic variability between RHDV isolates [3] and only one serotype had been described until Capucci et al. [7] and Schirrmeier et al. [27] identified RHDV antigenic variants in Italy and Germany, respectively. Given the genetic and antigenic differences between the variants and the original RHDV strains, the variants were considered to constitute a distinct subtype, designated by Capucci et al. as "RHDVa" [7]. A recent epidemiological survey showed that this RHDV variant first appeared in the central regions of Italy, before spreading to the northern and southern regions in 1997 [10]. Furthermore, it was identified in an outbreak that occurred in the USA in 2000 [10].

We continued the molecular epidemiological survey of French RHDV isolates done by Le Gall et al. in 1998 [14] to monitor the genetic variation and the appearance of RHDV variants in domesticated and wild rabbits. We analysed 104 new RHDV isolates collected between 1993 and 2000 including some isolates for which the clinical analysis indicated atypical RHD among which was one isolate obtained from an overseas French territory, the Reunion Island, in 2000. We used phylogenetic methods to analyse these isolates and to compare their nucleotide sequences with those of some RHDV isolates representative of the three genogroups identified previously and 14 RHDV sequences from the database. We

also compared the antigenic and genetic properties of some of the new isolates with those of the reference strains and carried out a protection study to confirm the identity of the French "RHDVa" variants.

Materials and methods

Virus samples

We collected 104 liver specimens containing RHDV, as determined by sandwich-ELISA [12, 15], from domesticated and wild rabbit from various regions of mainland France, between 1993 and 2000; one specimen was collected from an overseas French territory, the Reunion Island located in the Indian Ocean, near the East coast of South Africa (Fig. 1). Most of the isolates were collected by the Maine-et-Loire Veterinary Laboratory, France, and a few were collected by the AFSSA Institute. Some isolates were sent to the AFSSA Institute when there were atypical clinical signs of RHD. The liver samples were stored at −20 °C and were thawed at least once to obtain an exudate that was subsequently used for immunocapture (IC)-RT-PCR.

Oligonucleotide primers

cDNA was synthesised using oligo-dT as a primer. For the phylogenetic analysis, two PCR primers, p33 (sense) and p34 (anti-sense), were used to amplify a 501-bp fragment from the 3′ end of the RHDV gene encoding for VP60 (Table 1). The first half of each primer corresponded

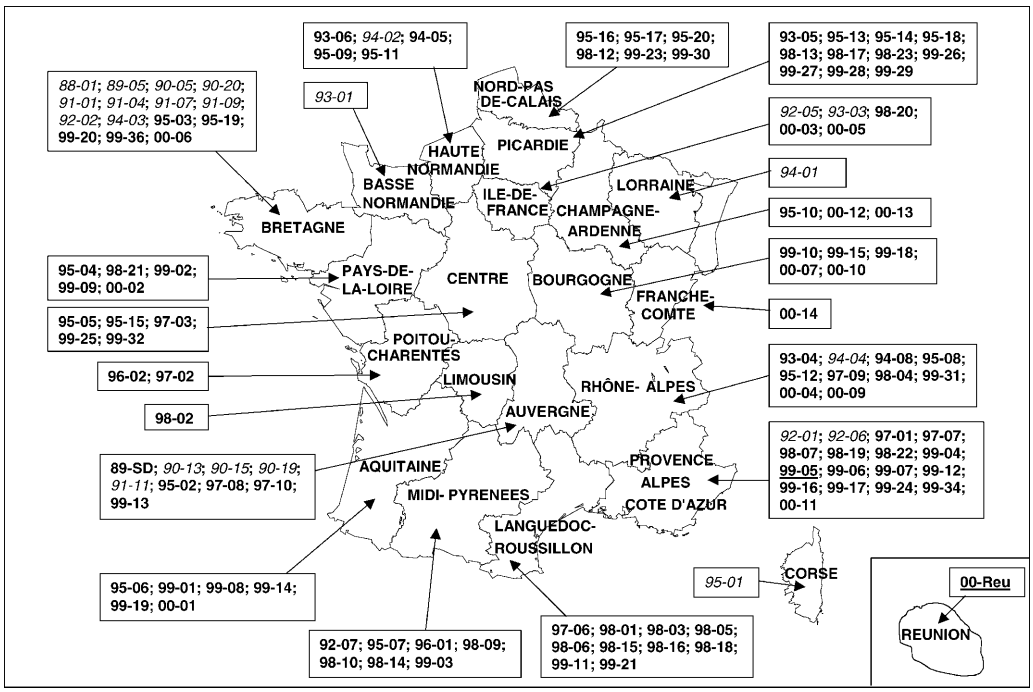


Fig. 1. Regions in France from which the sequenced RHDV isolates were recovered. The first two digits of the identification number correspond to the year of isolation and the last two digits to the collection order. The isolates 99-05 and 00-Reu are underlined. The RHDV isolates representative of the 3 genogroups identified previously [14] are in italics

Table 1. Primers used in this study

Primer	Sequence (5′–3′)[a]	Position (nt)[b]
p27[c]	ACATGTAAAACGACGGCCAGTCTCGGTAGTACCTGACGACG	5271–5290
p28[c]	CTGCAGGAAACAGCTATGACCGGGACGCAAGTCTGGCATGG	5810–5829
U351	GCAGTTTCGCTTCATAGT	5655–5672
L1303	TGGTTGGGGCGTGTATGT	6624–6607
824[d]	ATAGGCATTGGAACTTGA	6128–6145
937R[d]	GGCATACCAAAACTGGAG or GGCATACCAAAACTGAAG	6258–6241
p33[c]	ACATGTAAAACGACGGCCAGTCCACCACCAACACTTCAGGT	6473–6492
p34[c]	CTGCAGGAAACAGCTATGACCCAGGTTGAACACGAGTGTGC	6992–7011
RHDVAU	ACATGTAAAACGACGGCCAGTCCAGCCCACCACCAAYAC	6468–6485
RHDVAL	CTGCAGGAAACAGCTATGACCAAGTCCCAGTCCRATRAA	7046–7063

[a] M13 universal primer sequences are underlined
[b] The nucleotide positions refer to the RHDV-SD sequence (Accession number Z29514)
[c] Le Gall et al. [14]
[d] Sequencing primers only

to the standard sequencing primers (M13 forward and reverse primers, respectively) and the second half to the calicivirus-specific sequences [29].

The VP60 gene from three RHDV strains (99-05, 00-Reu and 00-13) was determined using three other pairs of primers to amplify three overlapping genomic regions (Table 1).

Immunocapture-RT-PCR

The IC-RT-PCR assay was performed as described by Le Gall-Reculé et al. [15]. Briefly, polystyrene microtitre plates (96-well plates) were coated with 100 µl of chicken anti-RHDV hyperimmune serum (diluted 1:1,000 in carbonate buffer, pH 9.6) and incubated for one hour or overnight at 37 °C. The wells were washed with PBS containing 0.2% Tween 20 (PBST). The liver exudate (100 µl diluted 1:2 in PBST) was added and incubated for one h at 37 °C. After washing, 25 µl of the reverse transcriptase mixture was added to each well and the plate was incubated at 37 °C for one h. We used 8 µl of each cDNA solution for the PCR. The reaction was accomplished in microtubes in a final volume of 20 µl with p33 and p34 primers.

The amplified PCR products (predicted size = 581 bp) were analysed by agarose gel electrophoresis [26] and purified (Geneclean II kit, BIO 101). The DNA sequence was determined by sequencing in both senses by the dye terminator method (ABI PRISM DyeTerminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems) on an automatic DNA sequencer ABI 373XL (Applied Biosystems) with the M13 forward and reverse universal primers.

Sequencing of the VP60 gene

For three RHDV strains, the cDNA obtained from the IC-RT assay was used for three different PCRs. A 521-bp fragment from the 5′ end of the gene was amplified as described by Le Gall-Reculé et al. [15] using the p27–p28 primer pair (Table 1). The 3′ end of the gene was amplified in a 20 µl volume containing 40 pmol of each primer (RHDVAU and RHDVAL) (Table 1) and 0.3 U of Tub polymerase (Amersham). After the denaturation step, five preliminary cycles of 30 sec at 92 °C, 30 sec at 54 °C and 45 sec at 72 °C were conducted, followed by 25 cycles of 30 sec at 92 °C, 30 sec at 64 °C and 45 sec at 72 °C and a final elongation step of

10 min at 72 °C. The region between these two regions was amplified in a 20 µl reaction mixture containing 10 pmol of each primer (U351 and L1303) (Table 1), and 0.3 units of Tub polymerase (Amersham). The PCR conditions were as follows: 92 °C for 30 sec, followed by 30 cycles of 30 sec at 92 °C, 30 sec at 55.3 °C and 1 min at 72 °C and a final elongation step of 10 min at 72 °C.

The amplified PCR products were analysed by electrophoresis on agarose gels [26] and purified. The nucleotide sequences of the 3′ end and 5′ end of the three RHDV genes encoding for VP60 were determined as mentioned above with the M13 forward and reverse universal primers. The nucleotide sequence of the inner region was determined with the corresponding PCR primers and the 824 and 937R (Table 1).

Sequence analysis

Multiple sequence alignments were carried out with the PILEUP program (Genetic Computer Group, Madison, WI, USA) and phylogenetic analyses were performed using the Phylogenetic interference package (PHYLIP) [8]. Phylogenetic relationships were inferred using i) the DNAPARS program, based on the principle of maximum parsimony and ii) the DNADist program (estimation of the distances between pairs of sequences) followed by UPGMA or neighbour-joining programs (building of the trees) based on the principle of phenetics. Bootstrap support percentages were calculated for each branching point of the tree by the Seqboot procedure (100 replicates) and the majority-rule consensus tree was determined by the CONSENSE program. Trees were plotted using DRAWGRAM. The sequence of the non-pathogenic RHDV strain, the rabbit calicivirus (RCV, Table 2), was used as an outgroup for the phylogenetic analysis.

The deduced amino acid sequences of the VP60 gene from the three RHDV strains were analysed using the software available on the Infobiogen web site (http://www.infobiogen.fr). Multiple sequence alignments were generated by the CLUSTAL method [28].

Antigenic characterisation of the RHDV isolates

The antigenicity of the isolates 99-05 and 00-Reu against various monoclonal antibodies (MAbs) from a MAb library raised against RHDV isolate 89-SD was undertaken [13]. Antigenicity was tested by capture sandwich ELISA. Purified recombinant RHDV-like particles (VLPs) produced in the baculovirus system [13] were used as a positive control and a liver exudate from a healthy rabbit was used as a negative control. Polystyrene microtitre plates (96-well plates) were coated with 100 µl of anti-RHDV MAb (ascitic fluid diluted 1:100 in PBS buffer) and incubated overnight at room temperature. The wells were washed with PBS containing 0.2% Tween 20 (PBST) and non-specific sites were saturated with PBS containing 1% gelatin for one hour at 37 °C. Serial two-fold dilutions of the antigen (VLPs diluted 1:600 and liver exudate diluted 1:12 in PBST for the initial dilution step) were made. After washing, the bound antigens were detected by sequentially adding convalescent rabbit serum SL10 [13] and an alkaline phosphatase anti-IgG conjugate (Sigma). The substrate used was 1 mg/ml pNPP (Sigma) in diethanol amine buffer. Optical density was read at 405 nm after 30 min at 37 °C.

The antigenic profiles of the two isolates were characterised in the O.I.E. (World Organisation of Animal Health) reference laboratory for caliciviruses of lagomorphs in Italy, by use of a panel of monoclonal antibodies for the antigenic characterisation of the "RHDVa" subtype [5, 7].

Transmission and protection studies

In a first experiment, an RHDV-seronegative 11-week-old rabbit was inoculated with 300 µl of 1/2 diluted liver exudate of RHDV isolate 9905 via the intramuscular route. The rabbit was

Table 2. Sources of RHDV available in database used in this study and the genogroup in which they were placed based on phylogenetic analysis

Country	Month/year collection	Region or city	Identification number (previous genogroup)	EMBL/GenBank/DDBJ accession number	Reference	Genogroup
Czechoslovakia	1987		87-TC (G2)	RH54983/U54983	Gould et al., 1997 [9]	G2
Germany	1989		89-GE (G2)	RHDVCG/M67473	Meyers et al., 1991 [17]	G2
Germany	1989	Eisenhüttenstadt	89-Eis	RHDVEI/Y15440	Schirrmeier et al., 1999 [27]	G1
Mexico	1989	Mexico	89-Mex	AF295785	Babcock et al., 2000 [2]	G2
France	1989	Auvergne	89-SD (G1)	RHDVCGS/Z29514	Rasschaert et al., 1994 [24]	G1
Spain	1989	Asturias	89-SP (G1)	RHDVGNS/Z49271	Boga et al., 1994 [4]	G1
Germany	1990	Hagenow	90-Hag	RHDVHAG/Y15441	Schirrmeier et al., 1999 [27]	G3
United Kingdom	1992	Ascot	92-UK (G3)		Le Gall et al., 1998 [14]	G3
Germany	1993	Meiningen	93-Mei	RHDVME/Y15426	Schirrmeier et al., 1999 [27]	G3
Germany	1996	Wriezen	96-Wri	RHDVWR/Y15427	Schirrmeier et al., 1999 [27]	G5
Germany	1996	Triptis	96-Tri	RHDVTR/Y15442	Schirrmeier et al., 1999 [27]	G6
Germany	1996	Frankfurt	96-Fra	RHDVFR/Y15424	Schirrmeier et al., 1999 [27]	G4
Germany	1996	Hartmannsdorf	96-Har	RHDVHA/Y15425	Schirrmeier et al., 1999 [27]	G6
USA	2000	Crawford County, Iowa	00-Iow	AF258618	Neilan et al., 2000 [19]	G6
Italy	1996	Brescia	RCV	RCVVP60/X96868	Capucci et al., 1996 [6]	/

housed in filtered-air room and observed for morbidity and mortality. The dead rabbit was examined for macroscopic lesions and a liver sample was collected.

In a second experiment, 50 RHDV-seronegative 8-week-old rabbits were divided into two groups of equal weight and housed in filtered-air rooms. Five rabbits from each group were immunised with a commercial inactivated vaccine (Lapinject®, Sanofi Santé Animale) according to the supplier's instructions (batch A). Five other rabbits from each group were not vaccinated (batch E) and the others received reduced doses of the vaccine (5 rabbits from each group received 1/4, 1/16 and 1/32 of the recommended dose, batches B, C and D respectively). Twelve days later, the rabbits in the first group were challenged by an intramuscular injection of 10^3 times the LD_{50} of the reference RHDV strain V/RHD/4, isolated in 1988 in France (88-01 isolate, Fig. 1). This strain is similar to the strains used in the French commercial vaccines. The rabbits in the second group were challenged by intramuscular injection with approximately the same lethal dose of RHDV isolate 99-05.

The rabbits were observed for morbidity and mortality: any dead rabbits were examined for macroscopic lesions and a liver sample was collected. All of the surviving rabbits were euthanised after 10 days. They were also examined for macroscopic lesions and liver samples were collected.

Results

Phylogeny of RHDV

A 501-bp fragment of the VP60 gene was amplified by IC-RT-PCR from all the liver specimens tested. All 104 fragments were sequenced and the sequences were aligned with the sequences of the homologous region of the VP60 genes from isolates representative of the three known genogroups (G1, G2 and G3; [14]), some reference strains (Table 2), and German and American antigenic RHDV variants (Table 2).

The cladistic and phenetic methods gave similar results. The 141 RHDV sequences were clustered into six major genogroups (G1 to G6, Fig. 2). As expected, the sequences representative of the old genogroups (G1, G2 and G3) were clustered into their own genogroups. The only exceptions were isolates 94-02 and 95-01 (underlined in the Fig. 2), which appeared to cluster in the new genogroups G5 and G4, respectively. Foreign reference strains were also found in the three old groups: strain Eisenhüttenstadt (89-Eis), the German vaccine strain isolated in 1989 was located in G1; the RHDV strain isolated in Mexico in 1989 (89-Mex) was located in G2; and the Hagenow (90-Hag) and Meiningen (93-Mei) strains isolated in 1990 and 1993 respectively were both located in G3. All of the new sequences were located in the three new genogroups (G4, G5 and G6), as supported by significant bootstrap values (58, 70 and 100%, respectively). Genogroup G4 clustered some of the RHDV isolates collected in France between 1993 and 1999 and the German strain isolated in Frankfurt in 1996 (96-Fra). Some of the RHDV isolates collected in France between 1994 and 2000, and the German strain isolated in Wriezen (96-Wri) in 1996 were clustered in genogroup G5. G5 included the most recent isolates from mainland France: all the isolates collected in 2000 and 83% of those isolated in 1998 and 1999. G6 clustered the isolates 99-05 and 00-Reu together with the German "RHDVa" antigenic variant

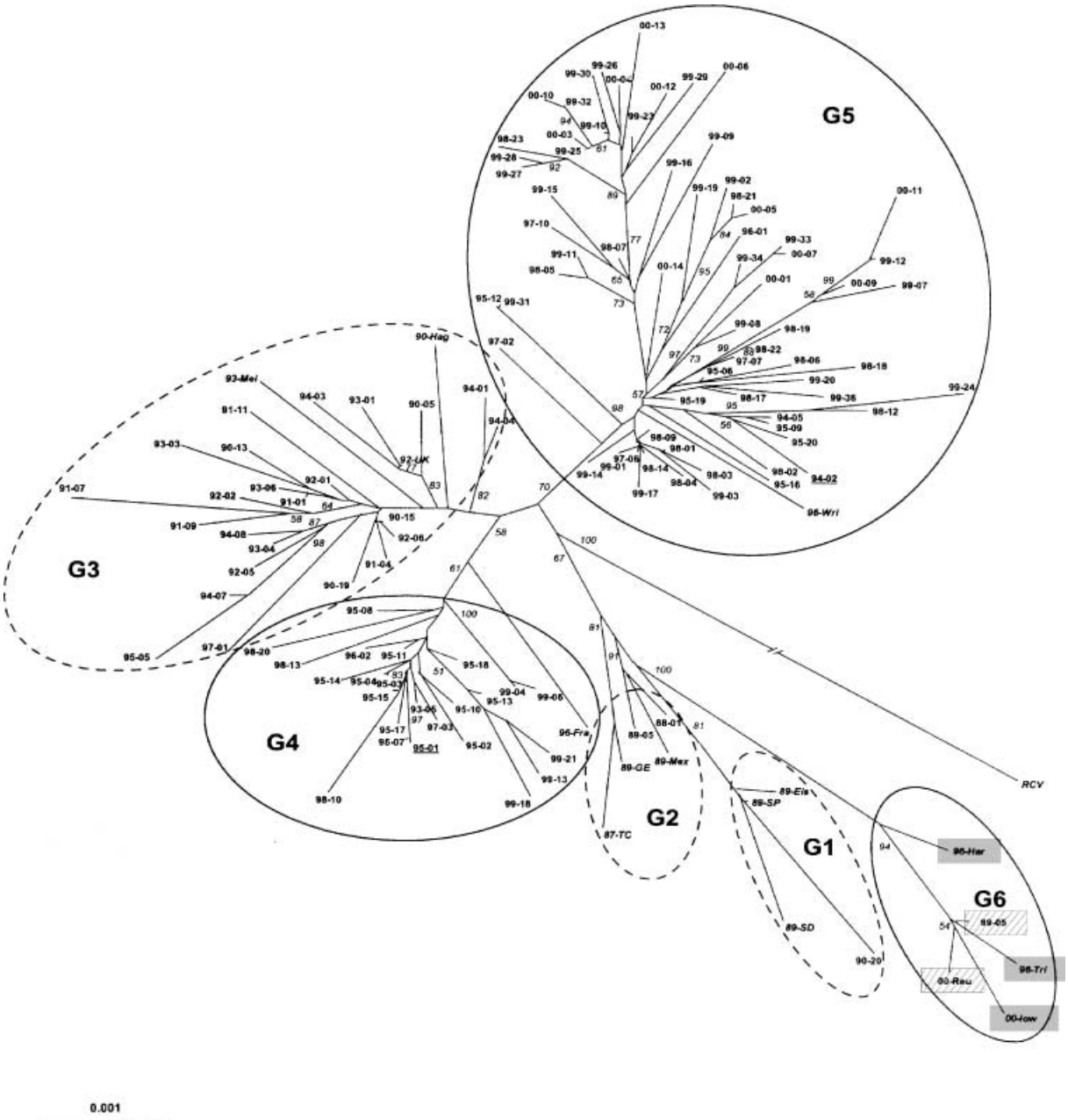


Fig. 2. Phylogenetic tree derived by use of the neighbour-joining method and 142 RHDV nucleotide sequences. Bootstrap values greater than 50% (for 100 replicates) are given in italics after each node. The branch lengths are proportional to the genetic distance and RCV was used as an outgroup to root the tree. For isolate identification number and origin, see Table 1. German and American "RHDVa" antigenic variants are highlighted in grey. The two French isolates 99-05 and 00-Reu are highlighted in grey hatched

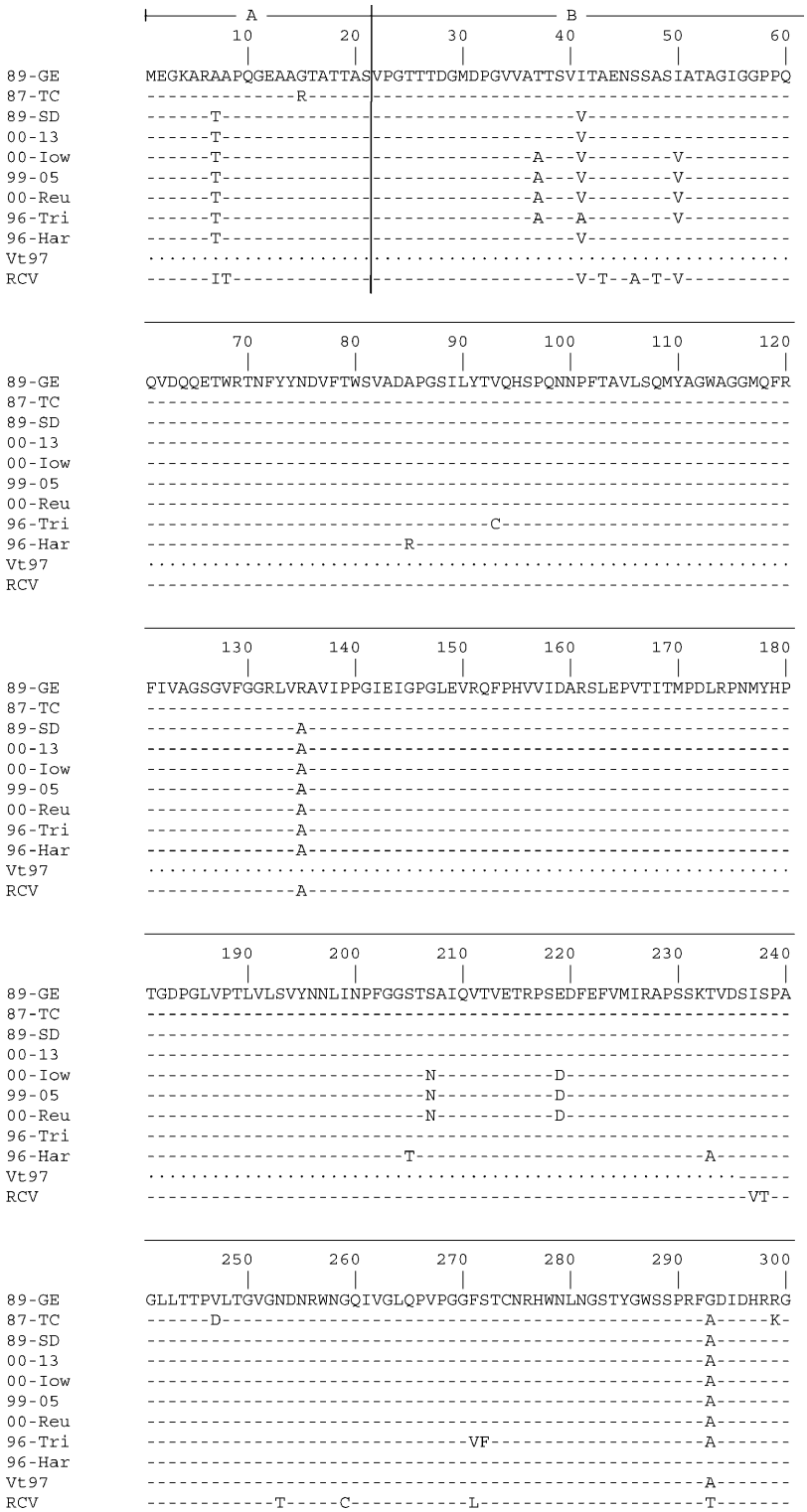
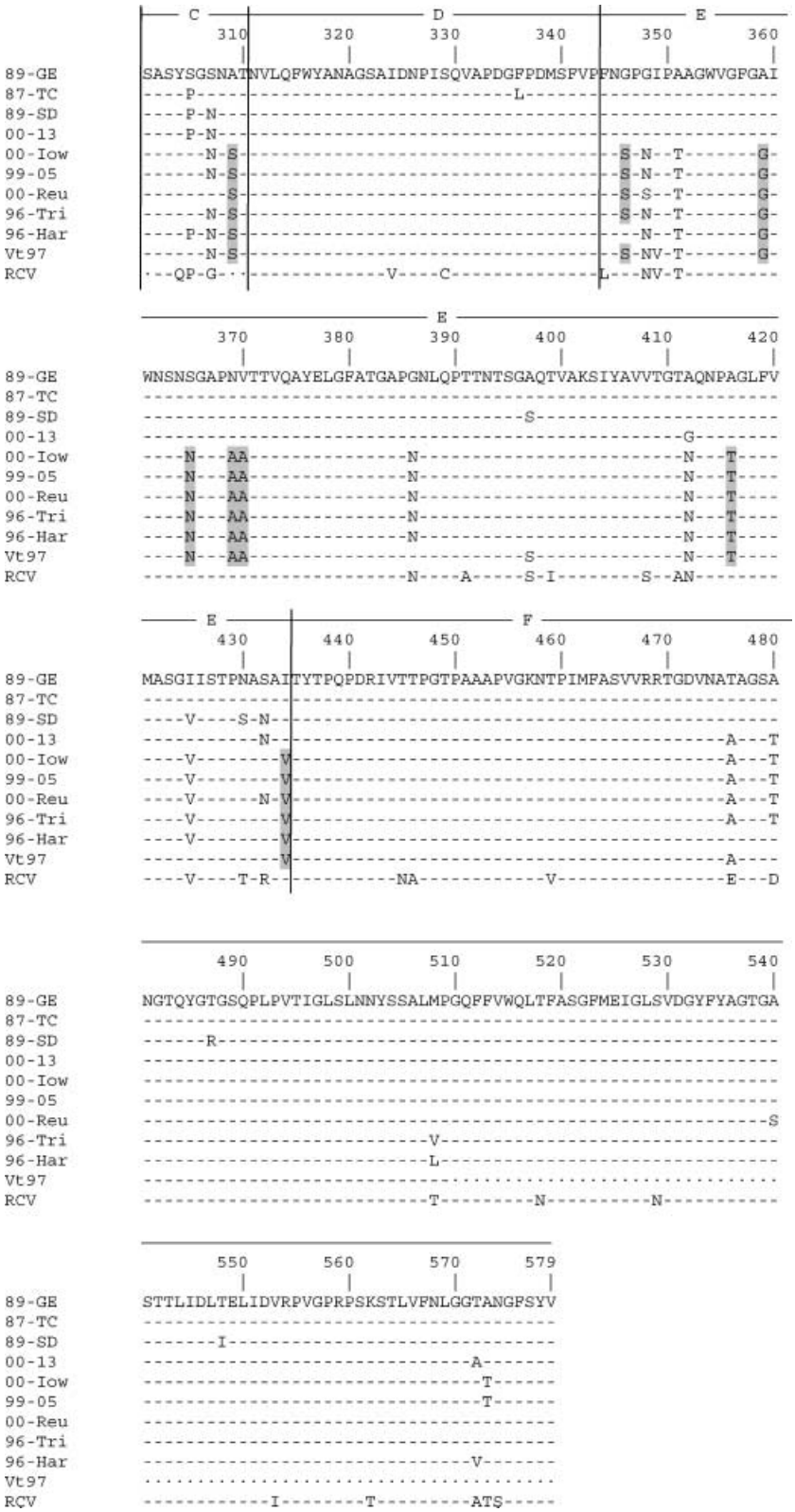


Fig. 3 (*continued*)


C
D
E
310 320 330 340 350 360
89-GE SASYSGSNATNVLQFWYANAGSAIDNPISQVAPDGFPDMSFVPPNGPGIPAAGWVGFGAI
87-TC ----P-----L-------
89-SD ----P-N-----
00-13 ----P-N-----
00-Iow ------N-S-----S-N--T-------G-
99-05 ------N-S-----S-N--T-------G-
00-Reu -------S-----S-S--T-------G-
96-Tri ------N-S-----S-N--T-------G-
96-Har ----P-N-S-----N--T-------G-
Vt97 ------N-S-----S-NV-T-------G-
RCV ---QP-G---V----C-----L---NV-T---------
E
370 380 390 400 410 420
89-GE WNSNSGAPNVTTVQAYELGFATGAPGNLQPTTNTSGAQTVAKSIYAVVTGTAQNPAGLFV
87-TC ----------
89-SD ----------S----------
00-13 ----------G---------
00-Iow ----N---AA----------N----------N---T----
99-05 ----N---AA----------N----------N---T----
00-Reu ----N---AA----------N----------N---T----
96-Tri ----N---AA----------N----------N---T----
96-Har ----N---AA----------N----------N---T----
Vt97 ----N---AA----------S----------N---T----
RCV ----------N----A-----S-I--------S--AN--------
E
F
430 440 450 460 470 480
89-GE MASGIISTPNASAITYTPQPDRIVTTPGTPAAAPVGKNTPIMFASVVRRTGDVNATAGSA
87-TC ----------
89-SD ----V----S-N-----
00-13 ----------N----------A---T
00-Iow ----V--------V----------A---T
99-05 ----V--------V----------A---T
00-Reu ----V------N-V----------A---T
96-Tri ----V--------V----------A---T
96-Har ----V--------V----------
Vt97 ----------V----------A----
RCV ----V----T-R-----NA----------V----------E---D
490 500 510 520 530 540
89-GE NGTQYGTGSQPLPVTIGLSLNNYSSALMPGQPFVWQLTPASGFMEIGLSVDGYFYAGTGA
87-TC ----------
89-SD ------R-----
00-13 ----------
00-Iow ----------
99-05 ----------
00-Reu ----------S
96-Tri ----------V----------
96-Har ----------L----------
Vt97 ----------
RCV ----------T---------N----------N----------
550 560 570 579
89-GE STTLIDLTELIDVRPVGPRPSKSTLVFNLGGTANGFSYV
87-TC ----------
89-SD -------I----------
00-13 ----------A-------
00-Iow ----------T------
99-05 ----------T------
00-Reu ----------
96-Tri ----------
96-Har ----------V-------
Vt97 ....
RCV ----------I---------T---------ATS-----

strains isolated in Triptis (96-Tri) and in Hartmannsdorf (96-Har) in 1996, and the American "RHDVa" strain (00-Iow) collected in Iowa in 2000.

The maximum nucleotide divergence was found between the isolates in genogroup G5 and those in genogroup G6 (up to 9.9%), despite the fact that they were isolated during the same period. However, the divergence between the G6 isolates and the French RHDV reference strain (89-SD) was only between 6.4% and 6.8%. Divergence was higher between 89-SD and the G5 isolates (up to 8.5%).

The fact that two RHDV strains (99-05 and 00-Reu) isolated in France clustered in the same genogroup as "RHDVa" variants prompted us to determine the amino acid sequences of these new isolates. We also determined the amino acid sequence of the VP60 protein from an isolate (00-13) representative of genogroup G5, which includes isolates that are currently found in France. The accession numbers of these 3 strains are AJ302016, AJ303106 and AJ495856 respectively. The deduced amino acid sequences were aligned with the sequences of an Italian "RHDVa" strain (Vt97, shown in [7]), German "RHDVa" strains isolated in Triptis (96-Tri) and in Hartmannsdorf (96-Har), some reference strains available in databases and RCV (Fig. 3). Pairwise sequence comparisons (Table 3) showed that the highest genetic correlation was between 99-05 and 00-Iow (similar sequences), and between 99-05/00-Iow and 00-Reu (99.1% similarity). These isolates shared between 96.9% and 98.6% similarity with 96-Tri, 96-Har and Vt97, but only between 90.8% and 96.2% similarity with the reference strains 89-SD, 87-TC, 89-GE, RCV and with 00-13 (Table 3). Most of the differences between amino acid sequences were located in the hypervariable regions (regions C and E, Fig. 3).

Antigenic characterisation of the RHDV isolates 99-05 and 00-Reu

The antigenic reactivity of RHDV isolate 99-05 was tested against monoclonal antibodies from a library raised against the RHDV.89-SD reference strain and compared to the reactivity of the purified rRHDV VLPs from isolate 89-SD in

Fig. 3. Amino acid sequence alignment of the VP60 capsid protein from three RHDV isolates characterised in this study (00-13, 99-05 and 00-Reu; accession numbers: AJ495856, AJ302016 and AJ303106, respectively), four RHDV antigenic variants ("RHDVa"), three reference RHDV strains and RCV. Isolate 00-13 is representative of the isolates currently found in France (genogroup G5). The four "RHDVa" isolates are Vt97, the Italian isolate [7]; 96-Tri and 96-Har, the German isolates collected in Triptis and Hartmannsdorf, respectively [27] and 00-Iow, the American isolate collected in Iowa in 2000 [19]. The three reference RHDV strains were from the Czech Republic (87-TC), Germany (89-GE) and France (89-SD). The EMBL/GenBank/DDBJ accession numbers of these sequences (except Vt97 not available in database) are shown in Table 2. Residues differing from the first sequence are shown and a dot corresponds to a deletion. The capsid protein was divided into regions according to the nomenclature of Neill [20] as indicated by the letters (*A* to *E*) at the top of the alignment. The mutated amino acids of the French RHDV isolates similar to the published RHDV antigenic variants only, are highlighted in grey

Table 3. Amino acid sequence similarities (%) derived from pairwise comparisons of the VP60 gene from selected RHDV

	89-GE	87-TC	89-SD	00-13	00-Iow	99-05	00-Reu	96-Tri	96-Har	Vt97*	RCV
89-GE	100	99.0	97.9	98.3	95.7	95.7	95.7	95.5	96.2	94.2	91.5
87-TC		100	97.6	97.9	95.0	95.0	95.0	94.8	95.5	93.1	91.0
89-SD			100	98.6	95.7	95.7	95.7	95.3	96.2	93.4	92.1
00-13				100	96.2	96.2	96.2	95.9	96.5	93.8	92.2
00-Iow					100	100	99.1	98.6	97.4	98.2	91.0
99-05						100	99.1	98.6	97.4	98.2	91.0
00-Reu							100	98.1	96.9	97.1	90.8
96-Tri								100	97.2	97.1	90.7
96-Har									100	96.7	91.4
Vt97*										100	86.1
RCV											100

*The partial VP60 gene sequence available in Capucci et al. [7] was used
The scores of the most closely related strains are boxed

a capture sandwich ELISA test. The same reaction pattern was obtained for the RHDV 99-05 exudate and for the rRHDV VLPs, whereas the exudate from healthy rabbit liver was not reactive against RHDV MAbs. Thus, the four tested RHDV neutralising MAbs (D23, E29, E3 and C36) recognised 99-05 as well as the rRHDV VLPs. However, when the antigenic profile of isolate 99-05 was determined by use of the Italian MAbs, it was not reactive in ELISA with 1H8 MAb which is able to protect experimentally infected rabbits from the disease [5]. Thus, its reactivity was similar to that of the Italian antigenic variants, "RHDVa" isolates Vt97 and Pv97 (Capucci, personal communication). Similarly, the isolate 00-Reu was not reactive with 1H8 MAb.

Transmission and protection studies with RHDV isolate 99-05

We characterised the necrotic lesions caused by isolate 99-05. In our experimental conditions, the inoculated rabbit died within 36 h. At necropsy, bloody exudate was found in the thoracic cavity and severe lesions were observed in the trachea, thymus, lungs, liver, spleen and kidneys. All of these organs were very congested. Moreover, haemorrhagic secretions were observed in the trachea, haemorrhages were noticed in lungs, the liver showed a reduced consistency and the spleen was enlarged.

We determined the degree of protection conferred by vaccination after a challenge with isolate 99-05 (Table 4). Unvaccinated control rabbits died within 36–60 h after challenge infection with the reference strain V/RHD/4, whereas all of the vaccinated rabbits survived. Three of the five unvaccinated rabbits challenged with 99-05 died within 36–60 h. The two surviving rabbits were RHDV-negative. All of the rabbits vaccinated with a dilution up to 1:16 survived the challenge infection. One of the five rabbits immunised with a vaccine dilution of 1:32 died 36 hours later and was RHDV-positive.

Table 4. Protection conferred against a challenge with our reference RHDV strain V/RHD/4 or RHDV isolate 99-05 after immunisation with normal and reduced doses of a commercial vaccine

Batch	Dose of vaccine	Challenge virus	Dead/total
A	1	V/RHD/4	0/5
		isolate 99-05	0/5
B	1/4	V/RHD/4	0/5
		isolate 99-05	0/5
C	1/16	V/RHD/4	0/5
		isolate 99-05	0/5
D	1/32	V/RHD/4	0/5
		isolate 99-05	1/5
E	unvaccinated control	V/RHD/4	5/5
		isolate 99-05	3/5

Discussion

The nucleotide sequences of 104 new isolates and 37 old isolates were subjected to phylogenetic analysis. Despite the low nucleotide sequence variation, six major genogroups (G1 to G6) were identified in the resulting trees. Our previous study identified three genogroups: G1, G2 and G3 [14]. Genogroups G1 and G2 clustered isolates collected between 1987 and 1990. These two groups were both present in France during this 3-year period. They were subsequently replaced by viruses that clustered in the genogroup G3 [14]. The representative sequences selected from these three genogroups were distributed in their own genogroups, except for isolates 95-01 and 94-02, which were found to be the prototypes of new genogroups G4 and G5, respectively. However, these two isolates were the most divergent isolates in the G3 group in the dendogram constructed by Le Gall et al. [14]. The high number of recent sequences analysed in the present study made it possible to cluster these isolates in two new genogroups. Genogroups G3 and G4 were not supported by high bootstrap values (45% and 58% respectively), whereas genogroup G5 was supported by a bootstrap value of 70%. Most of the mutational hot spots found in isolates belonging genogroup G4 were identical to those found in genogroups G1 and G3, whereas those that characterised genogroup G5 were distinct. These results suggest that genogroup G4 emerged from genogroup G3, which has now disappeared, and that genogroup G5 is a new independent group. This may also explain why genogroups G4 and G5 co-existed between 1994 and 1999. The fact that none of the isolates in genogroup G4 was collected after July 1999 implies that the viruses belonging to this group are going to disappear. The 50 isolates collected during the second six months of 2000 and the first seven months of 2001 (not included in this study) all clustered in genogroup G5, confirming this hypothesis.

We confirmed that RHDV is characterised by a succession of epizootics in rabbit populations, as previously described [14]. Indeed, French RHDV isolates

are distributed according to the year in which they were collected and not according to their geographical location. This result was confirmed by the fact that the European isolates collected between 1987 and 1996 were clustered according to their year of collection. Furthermore, we showed that there was little genomic variation within RHDV isolates over an 11-year-period. Comparison of nucleotide sequences indicated that 8.5% maximum divergence occurred between the more recent isolates in genogroup G5 and the French reference RHDV strain (89-SD), which is comparable to the 7.6% divergence that had occurred during the first 7 years [14].

Genogroup G6 is a special case. This genogroup clustered isolates with no apparent links (geographical or year of isolation). The isolate collected in mainland France in 1999 (99-05) and the isolate collected in a French overseas territory (the Reunion Island) in 2000 (00-Reu) were grouped with 96-Tri, 96-Har (Germany) and 00-Iow (USA), which are the new German and American RHDV antigenic variants [27, 10]. Two similar Italian variants were characterised by Capucci et al. [7] and named "RHDVa" subtype. The sequence of capsid proteins from isolates 99-05 and 00-Reu, and the antigenic profile of the Italian "RHDVa" showed that two "RHDVa" variants were isolated in France. Both French variants were very similar to each other and to the other "RHDVa" variants. The experimental infection of a susceptible rabbit showed that isolate 99-05 is highly pathogenic. However, the French commercial vaccine used in the vaccination assay protected rabbits from a challenge with 99-05. Schirrmeier et al. [27] showed that rabbits vaccinated with lower doses were less resistant to challenges, thus we tested several dilutions of the commercial vaccine. In our experimental conditions, all rabbits vaccinated with a dilution up to 1:16 resisted the challenge infection with isolate 99-05. Consequently, the currently available vaccine, produced from a strain isolated in 1989, fully protect rabbits from infection with 99-05. Furthermore, our neutralising monoclonal antibodies directed against the 89-SD strain (G1) recognised the 99-05 isolate as well as 89-SD, demonstrating that the neutralising epitopes of G6 group isolates are not significantly different to those of strains isolated in 1989.

The origin of these "RHDVa" variants is still unknown, but several hypotheses have been put forward. i) The nucleotide and amino acid sequence analyses showed that the variant isolates are distinct from RCV. This is especially true for the region within hypervariable region E of the VP60 gene which is thought to be responsible for modifying antigenic determinants (amino acids 344 to 370; [7]). This excludes the possibility that they appeared as a result of a recombination event between the two kind of viruses. ii) Schirrmeier et al. [27] isolated two antigenic variants, one from vaccinated stocks and the other from a six-week-old rabbit born of a vaccinated doe. This suggested that these two isolates were not neutralised by antibodies induced by vaccination. The two cases analysed in our study did not make it possible to confirm this hypothesis. Isolate 99-05 was collected during an outbreak of RHD among wild rabbits bred for hunting. In this case, a vaccinated breeder male and several 5-month-old rabbits died even though they were vaccinated after weaning. Conversely, the does that were vaccinated

twice a year, did not die, suggesting that the vaccination of the stock was imperfect. Moreover, our study showed that rabbits were protected by the vaccination against a challenge with the isolate 99-05. Isolate 00-Reu was sent to us because mortality was observed also in vaccinated does and it was impossible to stop the diffusion of the disease in spite of emergency vaccination of the breeding stock. These observations could suggest that it was an atypical strain of RHD. It was later shown that the vaccines used to immunise the rabbits were long out-of-date. Furthermore, we found only one "RHDVa" among 51 new isolates collected in mainland France until July 2001 [unpublished results]. This shows that this antigenic variant is rare in France. This observation is not consistent with the emergence of a variant that can escape vaccination. iii) We showed that genogroup G6 (clustering the antigenic variant isolates "RHDVa") is closely related to genogroup G1 (clustering the old strains and the German vaccine strain, 89-Eis). This result and the description of the RHD outbreaks suggest that the variant was introduced via the vaccine. However, the RHD outbreak that occurred in the USA in 2000 contradicts this hypothesis because vaccination is not authorised in the USA. iv) The epidemiological survey done by Grazioli et al. [10] demonstrated that the "RHDVa" subtype was found in central Italy in 1995–1996 before spreading rapidly north and south. It is present now in most parts of Italy and in some north regions, completely replaced the original virus. Conversely in France, the "RHDVa" variant is still rare. However, "RHDVa" 99-05 was collected in a region close to Italy (Provence-Alpes-Côte-d'Azur region) and we characterised recently a "RHDVa" variant isolated in an area in north east France, near from Germany (Lorraine region; data not shown). Thus, this subtype has begun to spread geographically in France and could constitute a new genogroup that would completely replace RHDV in the future.

Our results highlight the circulation of isolates different from those of the epizootic in progress, although these isolates remain rare. It would be interesting to continue this study to follow the evolution of "RHDVa" in France and to detect the arrival of new RHDV antigenic variants.

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