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Phylogenetic Analysis of Selected RHDV Strains on the Basis of a Fragment of the Gene Encoding C-Terminal End of VP60 Capsid Structural Protein

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Abstract

Phylogenetic analyses of RHDV provide information on the affinity of strains and point to evolutionary dependencies among them. The objective of the study was the phylogenetic analysis of six strains of RHD virus, including four Czech strains (CAMPV-351, CAMPV-561, CAMPV-562, CAMPV-558) and two French strains (Fr-1, Fr-2), on the basis of a fragment of the gene encoding C-terminal end of VP60 capsid structural protein. Phylogenetic analysis involved 25 sequences of RHDV homologues obtained from RHDV GenBank. The phylogenetic tree generated for 31 RHDV strains on the basis of a fragment of the gene encoding C-terminal end of VP60 capsid structural protein divided the strains analysed into four genetic groups (G1-G4), whereas the strains analysed were grouped in three genetic groups: G1 (CAMPV-351, CAMPV-562, CAMPV-558), G2 (Fr-1, Fr-2) and G3 (CAMPV-561). The phylogenetic analysis performed for Czech and French strains evidences that the strains feature different evolutionary paths and derive from European strains that caused foci of the plague in Germany and France. The obtained distribution of strains into four genetic groups testifies to their evolution, which is proved by group 4 gathering RHDVa strains.

Keywords: Rabbit haemorrhagic diseae virus (RHDV); Phylogenetic analysis; VP60

Introduction

RHD (Rabbit haemorrhagic disease) virus was first described in 1984 in China, where it caused viral haemorrhagic disease (VHD), which is very acute [1]. At present, the disease spread onto all continents, and is characterised with very high mortality, reaching up to 100%. So far, various hypotheses have been described as regards the spread of the rabbit haemorrhagic disease virus (RHDV) worldwide and in Europe. Furthermore, studies on samples collected from rabbits deceased in the years 1950-1970 in the United Kingdom have indicated the presence of anti-RHD antibodies in serum and the presence of genetic material of the RHD virus in bone marrow [22]. These results point to the occurrence of the RHD virus in Europe long before the outbreak of the disease caused by the virus in China.

Phylogenetic analyses and the study on phylodynamics of RHDV strains has been the subject of many publications [2,3,5,7-9,11-16,18-20,22-25,28-30]. The analyses performed indicate that RHDV strains are grouped depending on the time of strain isolation and on geographic location, and that the strains referred to as antigen variants RHDVa (new RHDVa subtype) are genetically distanced from the strains referred to as original RHDV subtype, which is manifested by formation of completely separate genetic groups. It must be pointed out that phylogenetic analyses of RHDV strains on the basis of a fragment of the gene encoding C-terminal end of VP60 capsid structural protein have so far been performed by researchers from France and Poland [16,18,28]. Phylogenetic studies by the French team [16,18], who performed the analysis of 56 and 104 French RHDV strains from the years 1988-1995 and 1993-2000, as well as strains obtained from GenBank (including 5 RHDVa strains), indicated the distribution of the strains analysed into 3 and 6 genogroups (G1-G3, G1-G6), which differentiated with the time of strain isolation; whereas five analysed RHDVa strains (99-05, 00-Reu, Triptis, Hartmannsdorf, Iowa) were grouped in G6. In turn, Polish studies performed on the basis of analogical fragment, and involving six RHDV strains originating from the Central Europe (Eisenhuttenstadt, Frankfurt, Rossi, V-411, 24/89, 1447/96), from the years 1989-2002, indicated strain distribution into two genetic groups, while strain distribution in the groups correlated with geographic location and the time of isolation [28].

The objective of the study was to perform phylogenetic analysis of six European strains of RHD virus, including four Czech strains (CAMPV-351, CAMPV-561, CAMPV-562, CAMPV-558) and two French strains (Fr-2, Fr-2), on the basis of a fragment of the gene encoding C-terminal end of VP60 capsid structural protein. The results of own studies were compared with 25 sequences of RHDV homologues obtained from GenBank.

Materials and Methods

RHDV strains

The study involved four Czech strains (CAMPV-351 from 1987 (passage from 2000), CAMPV-561 from 1996, CAMPV-562 from 1992, CAMPV-558 from 1988) and two French strains (Fr-1 from 1994, Fr-2 from 1992) (Table 2), prepared in the form of lyophylisate according to the procedure previously described by Fitzner et al. [6]. Czech strains originated from the Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute in Brno (Czech Republic), while French strains were obtained from CNEVA- Laboratoire Central de Recherches Avicole et Porcine, Poulfragan, from Dr J. P. Morisse.

Isolation of viral RNA

RNA of RHD virus was isolated from lyophylisates using the RNA set Total RNA (A&A Biotechnology, Poland) according to the provided protocol.

Reverse transcription (RT) reaction-cDNA synthesis

Complementary cDNA strand were obtained on a matrix of viral RNA, using reverse transcriptase enzyme (M-MLV Reverse

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Transciptase, Invitrogen, USA). 25 µl of the reaction mixture contained: 1.0 µl of specific antisense starter (P5) at 100 µM concentration (Metabion GmbH, Germany), 1.0 µl of dNTPs nucleotide blend at 25 mM concentration (Promega, USA), 0.5 µl of reverse transcriptase enzyme M-MLV RT (Invitrogen, USA), 2.0 µl of 5-fold concentrated RT-PCR buffer (Invitrogen, USA), 0.5 µl DTT 0.1 M (Invitrogen, USA), 1.0 µl RNase inhibitor RNase OUT (Invitrogen, USA), 14 µl of water for molecular biology (Eppendorf, Germany) and 5.0 µl of RNA of an appropriate RHD virus strain. Before reaction mixture was prepared, RNA of five tested RHDV strains was heated for 5 minutes at 65°C, and then stored on ice until the mixture was prepared. RT-PCR reaction was conducted in a T-gradient Thermocycler (Biometra, Germany) using the following temperature-time profile: 25^{0}C for 10 minutes, 37^{0}C for 60 minutes, 95^{0}C for 5 minutes and 4^{0}C for 1 minute. Resulting cDNA was stored at the temperature of 2-8°C for further analyses.

Starters

Starters suggested by Guittre [10] were used, based on complete sequence of the RHDV-FRG genome, developed by Meyers et al. [21], and allowing for amplification of a fragment of the gene encoding C-terminal end of VP60 capsid structural protein. Using starters: P6 (sense) 5'acccagtcaggcaccaggctg3' and P5 (antisense) 5'gcacctgcaagtccaatccg3', a 320bp fragment was amplified. Starter synthesis was performer by Metabion GmbH (Germany).

PCR

50 µl of the reaction mixture contained: 2.0 µl of starters (1.0 µl of each P6P5) at 10 µM concentration each (Metabion GmbH, Germany), 1.0 µl of dNTPs blend at 10 mM concentration (Promega, USA), 5.0 µl of 10-fold concentrated Taq Plus buffer (GenoPlast, Poland), 1.0 µl of Taq Plus DNA polymerase (GenoPlast, Poland), 1.0 µl of 10-fold concentrated Mg buffer (Promega, USA), 38.0 µl of water for molecular biology (Eppendorf, Germany) and 2.0 µl of cDNA of an appropriate RHD virus strain (added to the reaction at the end). RT-PCR reaction was conducted in a T-gradient Thermocycler (Biometra, Germany). The following temperature-time profile was used: preliminary denaturation 94^{0}C-2 minutes, 35 cycles involving: denaturations (94^{0}C-30 seconds), starter affixing (50^{0}C, 53^0 or 55^{0}C-depending on the strain-1 minute), chain elongation (72^{0}C-2 minutes), final elongation (72^0 for 5 minutes) and cooling the reaction mixture to 4^{0}C. Reaction products were stored at the temperature of 4^{0}C for further analyses.

Electrophoresis of PCR products

For the purpose of visualisation of PCR products, electrophoresis was performed in 1.5% agarose gel (Prona, USA) dyed with ethidium bromide (Fermentas, Lithuania). Molecular mass marker GeneRuler 100 and 50 (Fermentas, Lithuania) was used for evaluation of size of products. Electrophoretic separation was conducted in 1.0-fold

	No.	RHDV strain	Country of origin	Year isolated	GenBank Accesion Number
RHDV	1.	V-351	Czech Republic	1987 (passage from 2000)	In preparation for submission to GenBank
	2.	CAMPV-561	Czech Republic	1996	FJ231995
	3.	CAMPV-562	Czech Republic	1992	FJ231996
	4.	CAMPV-558	Czech Republic	1988	FJ231997
	5.	Fr-1	France	1994	FJ231998
	6.	Fr-2	France	1992	FJ231999
	7.	Eisenhuttenstadt	Germany	1989	Y15440
	8.	FRG		1989	M67473
	9.	Meiningen		1993	Y15426
	10.	Jena		1993	EF558576
	11.	Frankfurt		1996	Y15424
	12.	Wriezen		1996	Y15427
	13.	Haute Saone	France	1988	U49726
	14.	SD		1989	Z29514
	15.	95-05		1995	AJ535092
	16.	95-10		1995	AJ535094
	17.	99-05		2005	AJ302016
	18.	00-13		2000	AJ495856
	19.	00-REU		2000	AJ303106
	20.	03-24		2003	AJ969628
	21.	BS89	Italy	1989	X87607
	22.	Italy90		1990	EU003579
	23.	AST89	Spain	1989	Z49271
	24.	Rainham	United Kingdom	1993	AJ006019
	25.	WX/China/1984	China	1984	AF402614
	26.	Whn/China/01/05		2005	DQ069280
	27.	KV0801	Korea	2008	FJ212322
	28.	Bahrain	Bahrain	2001	DQ189077
	29.	Iowa 2000	USA	2000	AF258618
	30.	NY-01		2001	EU003581
	31.	UT-01		2001	EU003582
	32.	RCV	Italy	1996	X96868

Table 1: RHDV and RCV strains used for phylogenetic analysis.

concentrated TBE buffer, at room temperature, with current voltage of 100 V/cm of gel for 45 minutes, using a set for electrophoresis (Bio-Rad, Germany). Storage and interpretation of results was completed using a UV visualisation set (Vilber Lourmat, France).

Preparative amplification, purification and preparation of analysed fragments of RHD virus genome for sequencing

Following PCR results visualisation, mass PCR was performed along with electrophoretic separation, using conditions identical as those described above. Next, DNA isolation from gel was performed using a Gel OUT set (A&A Biotechnology, Poland) according to the manufacturer's recommended procedure. Such prepared samples were sent for automatic sequencing to Metabion GmbH, Germany, and to DNA Sequencing and Oligonucleotide Synthesis Laboratory of the Institute of Biochemistry and Biophysics at the Polish Academy of Sciences, Warsaw, Poland.

Comparative analysis of nucleotide sequences, creation of homology matrix and phylogenetic tree

The obtained nucleotide sequences of a VP60 protein-coding gene C-terminal region fragment from six tested RHDV strains (CAMPV-351, CAMPV-561, CAMPV-562, CAMPV-558, Fr-1, Fr-2) were compared to one another (alignment) and to 25 homologous sequences of RHDV obtained from GenBank, whereas RCV strain served for rooting of the phylogenetic tree Table 1. The comparative analysis of the sequences was performed in DNAMAN software version 5.2.10 (Lynnon BioSoft, Canada). On the basis of the comparison of the nucleotide sequences of RHDV strains in DNAMAN software homology matrices were created, the basis of which was formed by common elements between the two sequences compared. The values from the matrices created have been graphically transformed into phylogenetic trees revealing hypothetical affinity and evolutionary dependences among the strains analysed. The tree was created using the maximum likelihood (ML) model. Each of the phylogenetic trees generated was a rooted tree, and the out group was formed by the sequence of the RCV virus (X96868). Bootstrap method was used for the assessment of the phylogenetic tree generated by the software.

Results

PCR yielded amplification of 320bp long genome fragment which was subsequently sequenced. Obtained sequences of six analysed strains (CAMPV-351, CAMPV-561, CAMPV-562, CAMPV-558, Fr-1, Fr-2) of RHDV were submitted to GenBank (except for CAMPV-351, which has been prepared for submission) Table 1. Obtained nucleotide sequences of six strains (CAMPV-351, CAMPV-561, CAMPV-562, CAMPV-558, Fr-1, Fr-2) of RHDV were compared to 25 sequences of RHDV homologues (Table 1). The phylogenetic tree (Figure 1) generated for 31 RHDV strains on the basis of a fragment of the gene encoding C-terminal end of VP60 capsid structural protein divided the strains analysed into four genetic groups (G1-G4), whereas the strains

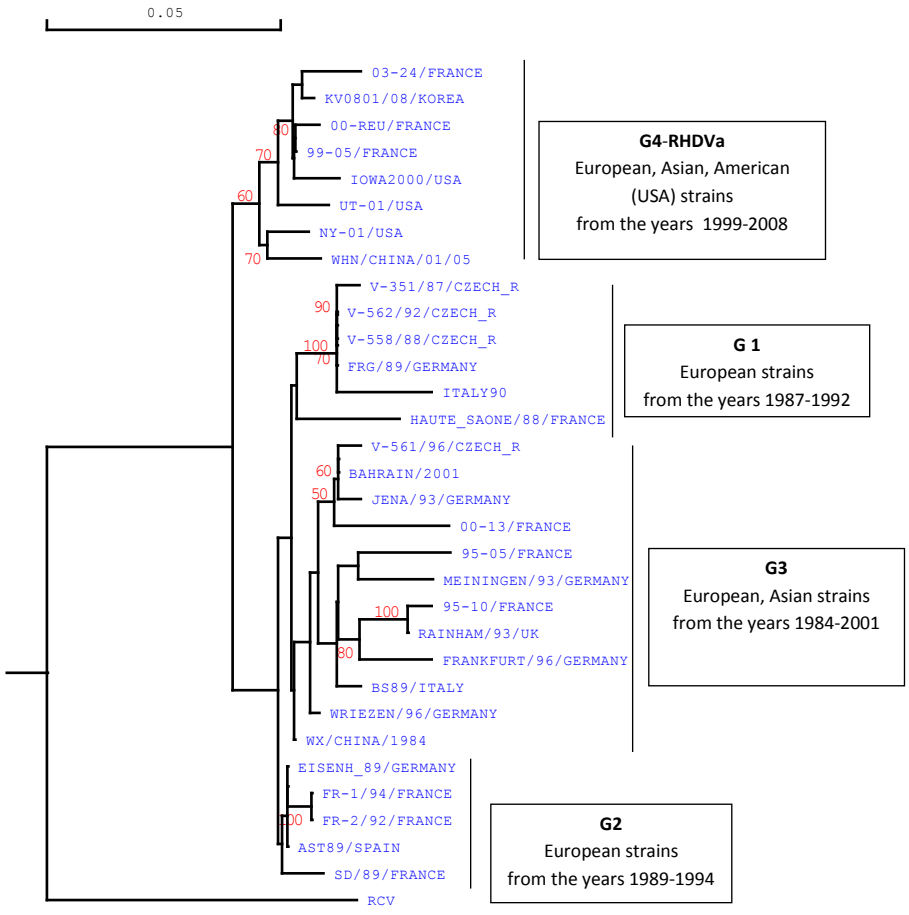

Figure 1: Phylogenetic tree on the basis of comparison of nucleotide sequences of the fragment of gene encoding C-terminal part of VP60 protein created with the maximum likelihood (ML) method.

	strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	**V-351/87/CZECH REP**	100	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
2	**V561/96/CZECH REP**	96.9	100																													
3	**V562/92/CZECH REP**	99.5	96.0	100																												
4	**V-558/88/CZECH REP**	99.5	96.5	100	100																											
5	**Fr-1/94/FRANCE**	97.4	97.1	97.9	97.9	100																										
6	**Fr-2/92/FRANCE**	97.4	97.4	97.9	97.9	100	100																									
7	00-13/FRANCE	94.8	96.9	95.4	95.4	95.4	95.3	100																								
8	03-24/FRANCE	93.8	93.8	94.3	94.3	95.4	95.3	91.8	100																							
9	00-REU/FRANCE	94.8	94.8	95.4	95.4	96.4	96.4	92.8	97.9	100																						
10	95-05/FRANCE	94.8	95.9	95.4	95.4	95.4	95.3	93.8	94.3	94.8	100																					
11	95-10/FRANCE	95.4	96.4	95.9	95.9	96.9	96.9	94.3	93.3	94.3	95.4	100																				
12	99-05/FRANCE	95.4	95.4	95.9	95.9	96.9	96.9	93.3	98.5	99.5	95.4	94.8	100																			
13	HAUTE SAONE/88/ FRANCE	96.4	95.9	96.9	96.9	96.4	96.4	94.8	94.8	94.8	95.4	94.3	95.4	100																		
14	SD/89/FRANCE	96.9	96.9	97.4	97.4	98.5	98.4	94.8	95.9	96.9	95.9	95.4	97.4	96.9	100																	
15	WX/CHINA/1984	95.5	98.5	99.0	99.0	99.0	99.0	96.4	95.4	96.4	96.4	96.9	96.9	97.4	98.5	100																
16	AST89/SPAIN	97.9	97.9	98.5	98.5	99.5	99.5	95.9	95.9	96.9	95.9	96.4	97.4	96.9	99.0	99.5	100															
17	BS89/ITALY	96.9	97.9	97.4	97.4	97.4	97.4	96.9	93.8	94.8	96.9	97.4	95.4	95.9	96.9	98.5	97.9	100														
18	ITALY90	97.4	95.4	97.9	97.9	95.9	95.9	93.3	94.3	94.3	93.3	93.8	94.8	94.8	95.4	96.9	96.4	95.4	100													
19	BAHRAIN/2001	97.4	99.5	97.9	97.9	97.9	97.9	97.4	94.3	95.4	96.4	96.9	95.9	96.4	97.4	99.0	98.5	98.5	95.9	100												
20	EISENH 89/ GERMANY	97.9	97.9	98.5	98.5	99.5	99.5	95.9	95.9	96.9	95.9	96.4	97.4	96.9	99.0	99.5	100	97.9	96.4	98.5	100											
21	FRANKFURT/96/ GERMANY	95.4	96.4	95.9	95.9	96.9	96.9	94.3	92.8	93.8	95.9	96.6	94.3	94.3	95.4	96.9	96.4	97.4	93.8	96.9	96.4	100										
22	FRG/89/GERMANY	99.5	97.4	100	100	97.9	97.9	95.4	94.3	95.4	95.4	95.9	95.9	96.9	97.4	99.0	98.5	97.4	97.9	97.9	98.5	95.9	100									
23	MEININGEN/93/ GERMANY	96.4	96.4	95.9	95.9	95.9	95.9	94.3	92.3	93.3	96.4	95.9	93.8	94.3	95.4	96.9	96.4	97.4	93.8	96.9	96.4	95.9	95.9	100								
24	JENA/93/ GERMANY	96.9	99.0	97.4	97.4	97.4	97.4	96.9	93.8	94.8	95.9	96.4	95.4	95.9	96.9	98.5	97.9	97.9	95.4	99.5	97.9	96.4	97.4	96.4	100							
25	WRIEZEN/96/ GERMANY	97.9	99.0	98.5	98.5	98.5	98.4	96.9	94.8	95.9	96.4	96.9	96.4	96.9	97.9	99.5	99.0	98.5	96.4	99.5	99.0	96.9	98.5	96.9	99.0	100						
26	IOWA2000/USA	94.3	94.3	94.8	94.8	95.9	95.9	93.3	97.4	98.5	94.3	93.8	99.0	94.3	96.4	95.9	96.4	94.3	93.8	94.8	96.4	93.3	94.8	93.3	94.3	95.4	100					
27	NY-01/USA	95.9	95.4	96.4	96.4	96.4	96.4	93.3	96.9	97.9	94.3	94.8	98.5	93.8	95.9	96.4	96.9	95.4	95.4	95.9	96.9	94.3	96.4	93.8	95.4	95.9	97.4	100				
28	UT-01/USA	94.8	94.8	95.4	95.4	96.4	96.4	92.8	97.4	97.9	94.8	93.3	98.5	94.8	96.9	96.4	96.9	94.8	94.3	95.4	96.9	93.8	95.4	93.3	94.8	95.9	97.4	96.9	100			
29	KV801/08/KOREA	94.8	94.8	95.4	95.4	96.4	96.4	92.8	98.5	99.0	95.9	94.3	99.5	95.4	96.9	96.4	96.9	94.8	94.3	95.4	96.9	93.8	95.4	93.3	94.8	95.9	98.5	97.9	97.9	100		
30	WHN/CHINA/01/05	95.4	94.8	95.9	95.9	96.4	96.4	92.8	96.9	97.9	93.8	94.3	98.5	94.8	95.9	96.4	96.9	94.8	94.8	95.4	96.9	93.8	95.9	93.3	94.8	95.9	97.4	97.9	96.9	97.9	100	
31	RAINHAM/93/UK	95.9	96.9	96.4	96.4	97.4	97.4	94.8	93.8	94.8	95.9	99.5	95.4	94.8	95.9	97.4	96.9	97.7	94.3	95.4	96.9	97.4	96.4	96.4	96.9	97.4	94.3	95.4	93.8	94.8	94.8	100

Table 2: Homology matrix for 31 RHDV strains on the basis of the fragment of gene encoding C-terminal part of VP60 protein, with the observed differentiation method.

analysed were grouped in three genetic groups: G1 (CAMPV-351, CAMPV-562, CAMPV-558), G2 (Fr-1, Fr-2) and G3 (CAMPV-561).

Genetic group one (G1) comprised just three European strains from the years 1987-1992, including the analysed Czech strains CAMPV-351, CAMPV-562 and CAMPV-V-558, as well as strains obtained from GenBank, namely German FRG, Italian ITALY90, and French HAUTE SAONE strain. Genetic group two (G2) also gathered European strains from the years 1989-1994, including the analysed French strains Fr-1 and Fr-2, as well as three strains obtained from GenBank, namely German Eisenhitennstadt, Spanish AST89, and French SD strain. The largest genetic group was group three (G3), which apart from the analysed Czech V-561 strain from 1996 included 11 strains from the years 1984-2001 obtained from GenBank, such as German: Jena93, Meiningen93, Frankfurt96, Wriezen96; French: 00-13, 95-05, 95-10; British Rainham; Italian BS89; as well as Bahrain and WX/China strains. Genetic group four (G4) comprised European, Asian and American strains from the years 1999-2008, namely eight strains, including three French strains: 03-24, 00-Reu, 99-05; three American: Iowa 2000, UT-01, NY-01; one Korean 0801/08, and one Chinese strain WHN. Bootstrap value in the tree generated amounted from 50% to 100%.

When analysing the homology matrix (Table 2) generated for 31 RHDV strains, it can be stated that homology determined on the basis of a fragment of the gene encoding C-terminal end of VP60 capsid structural protein among the strains in particular genetic groups was as follows: in G1: 94.8%-100%, in G2: 97.9-100%, in G3: 93.8-99.5%, while in G4: 96.9-99.5%. Furthermore, it was determined that strains V-562 and V-558 belonging to G1 revealed 100% sequence homology in the analysed gene fragment, as well as 100% homology as compared to the German FRG strain. In genogroup G2, also 100% homology was recorded for Fr-1 and Fr-2 strains; moreover, the strains revealed 99.5% homology with the Spanish AST89 strain. In turn, in genogroup G3, the highest homology of the nucleotide sequence, amounting to 99.5%, was observed for Czech CAMPV-561 strain as compared to Bahrain strain. In genogroup G4, the highest homology of 99.5% was recorded between KV0801 and 00-Reu strains on the one hand and 99-05 strain on the other, originating from Korea and France, respectively.

Discussion

The obtained distribution into genetic groups corresponds to the view according to which strains in the groups are linked by the time of their isolation rather than geographic origin [6,18,19]. In this respect, however, antigen variants differ, as they are always located in one genetic group [4,5,14,16,20,26]. In the case of genetic group one (G1), one can clearly define the criterion according to which the strains formed the group. It was formed of European strains from the Czech Republic, Germany, Italy and France, in the vast majority isolated in the years 1987-1989, namely upon the occurrence of the plague in Europe, with bootstrap value of 100%. This genetic group can be referred to as a "cluster" because it gathers strains having a common identification time, regardless of their geographic origin. Such positioning of strains in G1 corresponds to the results previously obtained by LeGall-Recule et al. [16,18], according to which strains in genogroups follow the time of isolation pattern, whereas strains isolated at a similar time are characterised by high homology. Detailed evolutionary relations in this group point to the fact that most strains in this group, except for French Haute Saone88 strain, derive from CAMPV-351, which is reflected in epizootic analysis of the plague in Europe in the years 1987-1989. A very similar relation can be observed in the case of G2, which comprised the analysed French Fr-1 and Fr-2 strains together with the strains isolated in 1989 in Spain and Italy (AST89 and SD/89). Evolutionary relations between the analysed Fr-1 and Fr-2 strains, supported with 100% homology between them (Table 2), indicate that these can be two "isolates" of the very same French strain, isolated in the same focus of the disease in the years 1992-1994. Genetic group three (G3) included strains from the years 1984-2001, which allows for suspecting that the evolutionary path of the Czech CAMPV-561 strain from 1996 led across Germany, and perhaps the strain derives from Jena strain from 1993 or Wriezen from 1996 (99.0% homology). Other strains in this group have common time of isolation. Strains originating from Europe, Asia and the USA, being antigen variants RHDVa (03-24, KV0801/08, 00-Reu, 99-05, Iowa, UT-01, NY-01,WHN) formed the fourth genetic group (G4) with homology ranging from 91.3% to 98.9%, which corresponds to genogroup 6 described by Le Gall et al. [18].

Conclusion

Since the identification of RHDV, its evolutionary paths have been traced, which is favoured by the emergence of increasingly new RHDV strains. Before, however, "new strains" appeared in the evolution tunnel, there must have been "older" strains from which it all started. According to one of the theories on virus evolution [27], RHDV strains will keep evolving, and this phenomenon will be manifested by new strains of the virus, which can nowadays be evidenced by French strain referred to as French RHD variant from 2010 [17], which is much different from the original RHDV subtype and the new RHDVa subtype, and its occurrence suggests differentiation of a new group of RHDV strains. Therefore, in view of these facts, it seems justified to analyse even the older RHDV strains from the years 1988-1996, as this allows for learning the true history of RHDV evolution, and perhaps will allow for analysing the evolution of RNA viruses. The phylogenetic analysis performed in own study for six RHDV strains, including four Czech (CAMPV-351, CAMPV-561, CAMPV-562, CAMPV-558) and two French (Fr-1, Fr-2) strains, on the basis of a fragment of the gene encoding C-terminal end of VP60 capsid structural protein, evidences that the strains feature different evolutionary paths and derive from European strains that caused foci of the disease in Germany and France. The obtained distribution of strains into four genetic groups testifies to their evolution, which is proved by group 4 gathering RHDVa strains.

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