Polish Journal of Microbiology
2009, Vol. 58, No 3, 237–245

ORIGINAL PAPER

Genetic Variability of Czech and German RHD Virus Strains

BEATA HUKOWSKA-SZEMATOWICZ* , MAŁGORZATA PAWLIKOWSKA
and WIESŁAW DEPTUŁA

Department of Microbiology and Immunology, Faculty of Natural Science, University of Szczecin, Poland

Received 22 June 2009, revised 15 July 2009, accepted 17 July 2009

Abstract

RHD (*rabbit haemorrhagic disease*) virus (RHDV) is the aetiological factor of the haemorrhagic disease of rabbits and is currently present on all continents. RHDV is a small, envelope-free virus containing genetic material in the form of a 7437-nucleotide long RNA strand. Studies indicate that genetic variability of RDHV strains originating from various parts of the world is approximately 14%, regardless the time and place of isolation. The aim of this study was to evaluate the genetic variability of 6 RHD virus strains from the Czech Republic (CAMPV-561, CAMPV-562, CAMPV-558) and Germany (Frankfurt, Wika, Rossi) based on analysis of fragment of a gene coding a non-structural p30 protein. The largest variability of nucleotide sequences within the studied fragment was found for the Rossi strain and CAMPV-562 (13.5%) and CAMPV-558 (13.5%), Wika and Frankfurt (12.1%), and CAMPV-561 and Wika (11.2%). Among the Czech strains the largest genetic distance was noted for strains CAMPV-558 and Iowa (0.130/0.140), and in the case of the German strains, for Frankfurt and Iowa (0.123/0.132). A homology tree constructed based on a fragment of a p30 protein-coding gene divided the 14 analysed strains into IV groups of 88% homology. Phylogenetic relationships also divided the tested strains into 4 genetic groups (G1-G4). The larger genetic distance exists between the Czech and German strains and the American ones, and the smallest between them and the European strains.

K e y w o r d s: RHD virus, genetic variability, phylogenetic analysis, polyprotein p30

Introduction

RHD (*rabbit haemorrhagic disease*) virus (RHDV) belongs to the *Caliciviridae* family and constitutes an aetiological factor of rabbit plague (rabbit haemorrhagic disease). The first outbreak of the disease caused by RHDV was reported in 1984 in China (Liu *et al.*, 1984). The first European foci of infection were noted in Italy in 1986, in former Czechoslovakia in 1987, in Germany in 1988, in France and Spain in 1989, and in Poland at the turn of 1987 and 1988 (Hukowska-Szematowicz, 2006). At present the disease has swept through all the continents.

RHDV is a small, envelope-free virus, 28–40 nm in size, with cubic symmetry. It contains a linear, single-stranded RNA made of 7437 nucleotides (Meyers *et al*., 1991, Meyers *et al.*, 2000; Wirblich *et al*., 1996). There are two reading frames in the RHD virus genome: a longer – ORF1 (7034 nucleotides), coding non-structural proteins, structural capsid protein VP60; and a shorter – ORF2 (353 nucleotides), coding a VP12 protein of yet unknown function (Meyers *et al*., 1991, Meyers *et al.*, 2000, Wirblich *et al*., 1996). Detailed genetic map of the RHD virus indicates within ORF1 sequences coding six non-structural proteins (p16, p23, p37 [helicase], p30, TCP [protease], polymerase), VPg protein and structural capsid protein VP60 in the following order: NH_2-p16-p23-p37(helicase)-p30-VPg-TCP(protease)-polymerase-Vp60-COOH (Wirblich *et al*., 1996). Currently, in the GenBank database (GenBank, 2009) there are 32 RHDV strains with completely sequenced genome (German, Spanish, French, Czech, British, New Zealand, American, Korean, Chinese, Japanese and from Bahrain and Saudi Arabia) and 37 RHDV strains with complete sequence of the structural capsid protein – VP60 (Chinese, Irish, French, Mexican, New Zealand, German, British and Korean). GenBank contains also over 200 registered RHD virus strains in which various fragments of their genome are sequenced, including a fragment coding the non-structural p30 protein. Long-term observations of course of the disease, and – most of all – results of

* Corresponding author: B. Hukowska-Szematowicz, Department of Microbiology and Immunology, Faculty of Natural Science, University of Szczecin, ul. Felczaka 3c, 71-412 Szczecin, Poland; phone (+48) 91 4441592; **e-mail: kurp13@univ.szczecin.pl**

RHD virus strains' tests performed using molecular biology methods (Asgari *et al*., 1999; Bildt *et al*., 2006; Capucci *et al*., 1998; Chrobocińska, 2007; Chrobocińska and Mizak, 2007; Farnos *et al*., 2007; Fitzner *et al.*, 2001, Fitzner, 2005; Fitzner and Kęsy, 2003; Forrester *at al*., 2006a; Forrester *at al*., 2006b; Gould *et al*., 1997; Hukowska-Szematowicz, 2006; Gall Le *et al.*, 1998; Gall-Recule Le *et al*., 2003; Matiz *et al.*, 2006; Milton *et al.*, 1992; Moss *et al*., 2002; McIntosh *et al.*, 2007, Nowotny *et al.*, 1997; Niedźwiedzka-Rystwej *et al*., 2009; Pawlikowska *et al*., 2009; Rasschaert *et al.*, 1994; Schirrmeier *et al.*, 1999; Tian *et al.*, 2007) show low level of genetic variability of RHDV of approximately 14%. According to Fitzner and Kęsy (2003), significant evolutionary conservation of the RHD virus genome is associated, among others, with rapid course of the disease and very frequent exchange of generations of susceptible rabbits. Both factors do not facilitate fixation of phenomena leading to the virus variability.

The aim of this study was to evaluate the genetic variability of 6 strains of RHD virus from the Czech Republic (CAMPV-561, CAMPV-562, CAMPV-558) and Germany (Frankfurt, Wika, Rossi) based on analysis of fragment of a gene coding the non-structural p30 protein.

Experimental

Materials and Methods

RHD virus strains. Three Czech strains were used (CAMPV-561, CAMPV-562, CAMPV-558) identified in 1988–1996, prepared in freeze-dried form according to a procedure described previously by Fitzner (Fitzner *et al*., 1996). Moreover, 3 German strains (Rossi, Wika, Frankfurt) from 1996–2002 were used, obtained from the liver of experimentally infected rabbits (Table I).

Isolation of viral RNA. Complete RNA of RHD virus was isolated from lyophilisates (in the case of Czech strains) and from 30% liver homogenates in buffered normal saline (in the case of German strains) 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 Transcriptase, Invitrogen, USA). 25 µl of the reaction mixture contained: 1.0 µl of specific antisense starter (C2) at 100 mM 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 six tested RHDV strains was heated for 5 minutes at 65°C, and then stored on ice until the mixture was prepared. RT-PCR was conducted in a T-gradient Thermocycler (Biometra, Germany) using the following temperature-time profile: 25°C for 10 minutes, 37°C for 60 minutes, 95°C for 5 minutes and 4°C for 1 minute. Resulting cDNA was stored at 2–8°C for further analyses.

Starters. Starters suggested by Guittre (Guittre *et al*., 1996) based on complete sequence of the RHDV-FRG virus genome, developed by Meyers

Table I
List of studied RHD virus strains and RHD reference strains obtained from GenBank

Strain	Year of identification	Country	GenBank accession number
CAMPV-561	1996	Czech Republic	FJ232000
CAMPV-562	1992	Czech Republic	FJ232001
CAMPV-558	1988	Czech Republic	FJ232002
WIKA	1996	Germany	in preparation for submission to GenBank
FRANKFURT	1996	Germany	in preparation for submission to GenBank
ROSSI	2002	Germany	in preparation for submission to GenBank
IOWA	2000	USA	AF 258618
NY-01	2001	USA	EU 003581
BAHRAIN	2000	Bahrain	DQ189077
pJG-DD06	2006	Germany	EF363035
BS	1989	Italy	X87607
SD	1989	France	Z29514
FGR	1988	Germany	M67473
V-351	1987	Czech Republic	U54983

(Meyers *et al*. 1991), and allowing amplification of fragment of the p30 protein-coding gene was used. Using starters: C1 (sense) 5'gttcacatatcgagggcgag 3' and C2 (antisense) 5'gacagggtcccttgagtacc3' a 490-bp fragment was amplified. Starter synthesis was performed by Metabion GmbH (Germany).

PCR. 50 µl of reaction mixture contained: 2.0 µl of starters (1.0 µl of each C1 and C2) at 10 mM concentration each (Metabion GmbH, Germany), 1.0 µl of dNTPs blend at 10 mM concentration (Promega, USA), 5.0 µl of 10-fold concentrated PCR buffer, 1.0 µl of Taq DNA polymerase, 1.0 µl of 10-fold concentrated buffer for Taq DNA polymerase (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 mixture in the end). PCR was conducted in a T-gradient Thermocycler (Biometra, Germany). The following temperature-time profile was used: preliminary denaturation 94°C – 2 minutes, 35 cycles involving denaturation (94°C – 30 seconds), starter affixing (50°C, 53°C or 55°C – depending on a strain – 1 minute), chain elongation (72°C – 2 minutes), final elongation (72°C for 5 minutes) and cooling the reaction mixture down to 4°C. Reaction products were stored at 4°C for further analyses.

Electrophoresis of PCR products in agarose gel. Electrophoresis in 1.5% agarose gel (Prona, USA) dyed with ethidium bromide (Fermentas, Lithuania) was performed in order to visualise PCR products. 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 concentrated TBE buffer, at room temperature, with current voltage of 100V/cm of gel for 45 minutes, using a set for electrophoresis from 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 to those described above. Preliminary DNA isolation from gel was performed using a Gel OUT set (A&A Biotechnology, Poland) according to the manufacturer's recommended procedure. Obtained samples were sent for automatic sequencing to Metabion GmbH, Germany and to the DNA Sequencing and Oligonucleotide Synthesis Laboratory at IBB PAN in Warsaw.

Molecular phylogenetic analysis of RHD virus sequence. Nucleotide sequences of a p30 protein-coding gene fragment from six tested RHDV strains obtained from sequencing were compared to each other and to eight homologous sequences obtained from Gene Bank (Table I). Sequence comparative analysis was performed using DNAMAN software, version 5.2.10 (Lynnon BioSoft, Canada). Based on comparison of nucleotide sequences of RHD virus strains matrices of distance and homology were constructed in DNAMAN software, constituting a method of transformation of biological sequences into mathematical data. The analysis used two methods of data transformation: observed divergence and maximum likelihood. The values of the resulting matrices were then graphically transformed into homology trees showing genetic relationships between analysed RHDV strains and expressed in %. Phylogenetic trees presenting hypothetical relationships and evolutionary relations between the analysed strains were constructed using observed divergence and maximum likelihood methods. A bootstrap method was used for evaluation of a program-generated phylogenetic tree. The method reports frequency of a given node occurrence in thousand newly-constructed trees (Baxevanis and Ouellette, 2004).

Results

PCR yielded amplification of 490-bp-long genome fragment which was subsequently sequenced. Obtained nucleotide sequences of 6 strains (CAMPV-561, CAMPV-562, CAMPV-558 and Wika, Rossi, Frankfurt) were compared to each other (a fragment of 430 nucleotides was compared) and to 8 homologous sequences obtained from GenBank (Table I).

Variability of nucleotide sequence in the tested p30 protein-coding gene fragment for the German and Czech strains was 5.7%. Genetic divergence of the tested Czech and German strains was manifested by the occurrence of 81 polymorphic loci, with repeated transitions and few transversions. The highest heterogeneity of nucleotide sequence within the tested fragment was observed for the Rossi strain and CAMPV-562 and CAMPV-558 (13.5%), Wika and Frankfurt (12.1%), CAMPV-561 and Wika (11.2%), and the lowest was for CAMPV-562 and CAMPV-558 (2.1%), Frankfurt and CAMPV-561 (2.8%) and Wika and Rossi (3%). In turn, comparative analysis of three tested Czech strains and three German strains to 8 strains originating from USA, Bahrain, Germany, Italy, France and the Czech Republic showed variability as the level of 5.5%. Nucleotide sequence comparison between 14 RHDV strains indicated 96 polymorphic loci with transitions dominating over transversions; no deletions or insertions were noted.

Constructed distance matrix (Table II) (presenting genetic distance for all sequence pairs in the set of analysed strains) showed that among the Czech strains

Table II
Distance matrix for 14 RHD virus strains performed using the observed divergence method (A) and the maximum likelihood method (B)

Strain	FRG	BAHRAIN	V-351	BS	SD	NY-01	IOWA	JPG-DD06	CAMPV-558	CAMPV-561	CAMPV-562	FRANK-FURT	ROSSI	WIKA
A.														
FRG	0	–	–	–	–	–	–	–	–	–	–	–	–	–
BAHRAIN	0.074	0	–	–	–	–	–	–	–	–	–	–	–	–
V-351	0.005	0.074	0	–	–	–	–	–	–	–	–	–	–	–
BS	0.063	0.053	0.058	0	–	–	–	–	–	–	–	–	–	–
SD	0.067	0.072	0.063	0.056	0	–	–	–	–	–	–	–	–	–
NY-01	0.119	0.107	0.119	0.109	0.102	0	–	–	–	–	–	–	–	–
IOWA	0.128	0.121	0.128	0.123	0.116	0.023	0	–	–	–	–	–	–	–
JPG-DD06	0.084	0.047	0.079	0.049	0.079	0.116	0.130	0	–	–	–	–	–	–
CAMPV-558	0.021	0.084	0.016	0.067	0.072	0.121	0.130	0.088	0	–	–	–	–	–
CAMPV-561	0.067	0.026	0.067	0.037	0.070	0.105	0.119	0.035	0.077	0	–	–	–	–
CAMPV-562	0.019	0.084	0.014	0.067	0.072	0.121	0.130	0.088	0.021	0.077	0	–	–	–
FRANKFURT	0.067	0.044	0.063	0.023	0.056	0.109	0.123	0.040	0.072	0.028	0.072	0	–	–
ROSSI	0.133	0.123	0.133	0.128	0.114	0.033	0.033	0.121	0.135	0.123	0.135	0.128	0	–
WIKA	0.121	0.114	0.121	0.116	0.114	0.021	0.016	0.119	0.123	0.112	0.123	0.121	0.030	0
B.														
FRG	0	–	–	–	–	–	–	–	–	–	–	–	–	–
BAHRAIN	0.078	0	–	–	–	–	–	–	–	–	–	–	–	–
V-351	0.005	0.078	0	–	–	–	–	–	–	–	–	–	–	–
BS	0.065	0.055	0.060	0	–	–	–	–	–	–	–	–	–	–
SD	0.070	0.075	0.065	0.058	0	–	–	–	–	–	–	–	–	–
NY-01	0.127	0.114	0.127	0.116	0.109	0	–	–	–	–	–	–	–	–
IOWA	0.138	0.130	0.138	0.133	0.125	0.023	0	–	–	–	–	–	–	–
JPG-DD06	0.087	0.048	0.082	0.050	0.082	0.124	0.140	0	–	–	–	–	–	–
CAMPV-558	0.021	0.088	0.016	0.070	0.075	0.129	0.140	0.093	0	–	–	–	–	–
CAMPV-561	0.070	0.026	0.070	0.038	0.072	0.111	0.127	0.035	0.080	0	–	–	–	–
CAMPV-562	0.019	0.088	0.014	0.070	0.075	0.130	0.140	0.093	0.021	0.080	0	–	–	–
FRANKFURT	0.070	0.045	0.065	0.023	0.057	0.116	0.132	0.040	0.075	0.028	0.075	0	–	–
ROSSI	0.144	0.133	0.144	0.138	0.122	0.033	0.033	0.130	0.146	0.132	0.146	0.138	0	–
WIKA	0.130	0.122	0.130	0.125	0.122	0.021	0.016	0.127	0.132	0.119	0.132	0.130	0.031	0

Table III
Homology matrix for 14 RHD virus strains performed using the observed divergence method

Strain	FRG	BAHRAIN	V-351	BS	SD	NY-01	IOWA	JPG-DD06	CAMPV-558	CAMPV-561	CAMPV-562	FRANK-FURT	ROSSI	WIKA
FRG	100%	–	–	–	–	–	–	–	–	–	–	–	–	–
BAHRAIN	92.6%	100%	–	–	–	–	–	–	–	–	–	–	–	–
V-351	99.5%	92.6%	100%	–	–	–	–	–	–	–	–	–	–	–
BS	93.7%	94.7%	94.2%	100%	–	–	–	–	–	–	–	–	–	–
SD	93.3%	92.8%	93.7%	94.4%	100%	–	–	–	–	–	–	–	–	–
NY-01	88.1%	89.3%	88.1%	89.1%	89.8%	100%	–	–	–	–	–	–	–	–
IOWA	87.2%	87.9%	87.2%	87.7%	88.4%	97.7%	100%	–	–	–	–	–	–	–
JPG-DD06	91.6%	95.3%	92.1%	95.1%	92.1%	88.4%	87.0%	100%	–	–	–	–	–	–
CAMPV-558	97.9%	91.6%	98.4%	93.3%	92.8%	87.9%	87.0%	91.2%	100%	–	–	–	–	–
CAMPV-561	93.3%	97.4%	93.3%	96.3%	93.0%	89.5%	88.1%	96.5%	92.3%	100%	–	–	–	–
CAMPV-562	98.1%	91.6%	98.6%	93.3%	92.8%	87.9%	87.0%	91.2%	97.9%	92.3%	100%	–	–	–
FRANKFURT	93.3%	95.6%	93.7%	97.7%	94.4%	89.1%	87.7%	96.0%	92.8%	97.2%	92.8%	100%	–	–
ROSSI	86.7%	87.7%	86.7%	87.2%	88.6%	96.7%	96.7%	87.9%	86.5%	87.7%	86.5%	87.2%	100%	–
WIKA	87.9%	88.6%	87.9%	88.4%	88.6%	97.9%	98.4%	88.1%	87.7%	88.8%	87.7%	87.9%	97.0%	100%

the largest genetic distance exists between strain pairs CAMPV-558 and Iowa (0.130 according to the observed divergence method/0.140 according to maximum likelihood method), CAMPV-558 and NY-01 (0.121/0.129); between CAMPV-561 and Iowa (0.119/0.127), CAMPV-561 and NY-01 (0.105/0.111) and between CAMPV-562 and Iowa (0.130/0.140). In turn, among German strains the highest distance was noted between strains: Frankfurt and Iowa (0.123/0.132), Frankfurt and NY-01 (0.109/0,116) and clear distance separates Rossi and Wika strains from the majority of the tested strains (0.114/0.119–0.135/0.146) (Table II), except for NY-01 (0.021–0.033) and Iowa (0.016–0.033), for which the distance was much smaller.

Constructed homology matrix for 14 RHDV strains (Table III) showed over 97% homology between strains, including CAMPV-562 and V-351 (98.6%), CAMPV-558 and V-351 (98.4%), Wika and Iowa (98.4%), CAMPV-562 and FRG (98.1%), CAMPV-558 and FRG (97.9%), Wika and NY-01 (97.9%), Frankfurt and BS (97.7%), CAMPV-561 and Bahrain (97.4%), Frankfurt and CAMPV-561 (97.2), Wika and Rossi (97%).

Homology tree (Figure 1) constructed based on fragment of a p30 protein-coding gene divided 14 analysed strains into IV groups (GI-GIV) of 88% homology. First one (GI) (with 98% homology) included two tested Czech strains: CAMPV-562, CAMPV-558 and FRG and V-351. The second group (GII) was formed solely by the French strain SD. The tested Czech strain CAMPV-561 and the German Frankfurt formed the third group (GIII), along with strains: Bahrain, BS and JPG-DD06 (96% homology). The fourth group (GIV) was composed of the tested German strains Wika and Rossi, and NY-01, Iowa (97% homology).

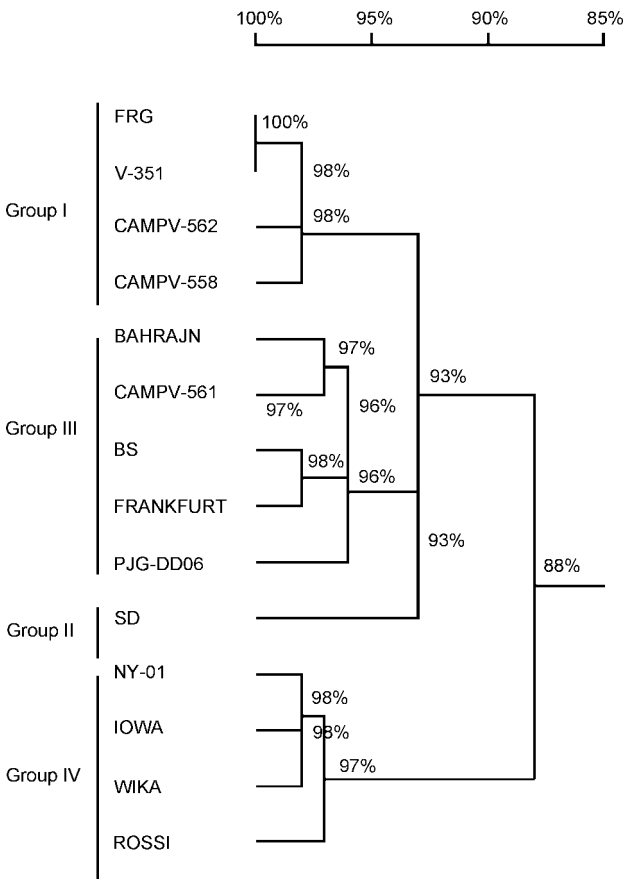

Fig. 1. Homology tree for 14 RHD strains performed using the observed divergence method.

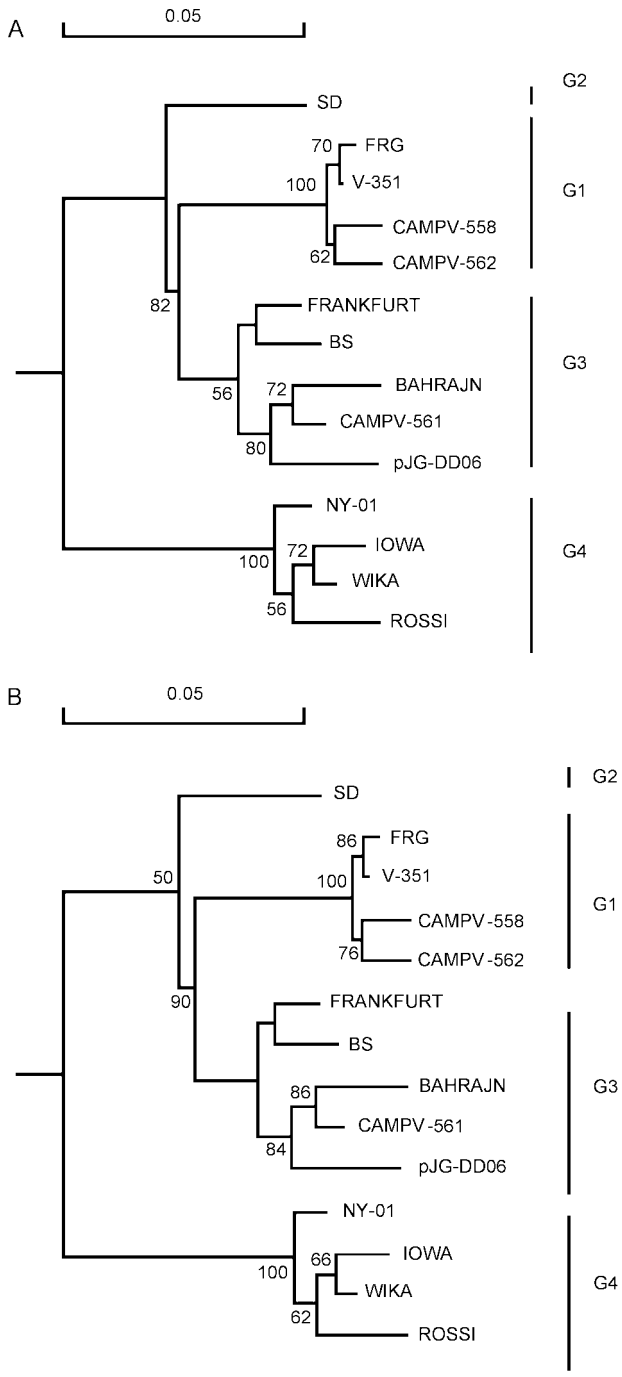

Fig. 2. Phylogenetic tree for 14 RHD virus strains performed using the observed divergence method (A) and the maximum likelihood method (B).

Phylogenetic relationships presented in the tree (Figure 2) also divided tested strains into 4 genetic groups (G1-G4), corresponding to the groups in homology tree (Figure 1). The first genetic group (G1) included strains: FRG, V-351, CAMPV-558 identified during the first years (1987–1988) of the plague development in Europe (Germany, Czech Republic), and the strain CAMPV-562 (1992), position of which on the tree may suggest that it comes from the strain CAMPV-558. Group G2 was formed by the French strain SD (1989), which appeared to belong to the G1 group along with the strains identified in 1980s. The third genetic group (G3) gathered strains from 1996–2006, except for the Italian BS strain (1989). Evolutionary relationships in that group indicate that the Bahrain strain (2000) could come from European strains. Group G4 included tested German strains Wika and Rossi (1996) and American: NY-01 (2001) and Iowa (2000), constituting antigenic variants of RHDV. Topology of phylogenetic trees constructed both using observed divergence and maximum likelihood were very similar, and the bootstrap value was higher in case of the highest likelihood method, equal to 50–100, confirming very high reliability of the generated phylogenetic tree.

Discussion

The variability of nucleotide sequences within the compared sequences of Czech and German strains (5.7%) and variability of tested strains and those obtained from the GenBank (5.5%) noted in this study is similar to the variability noted by other researchers (0–14.0%), analysing sequence variability within the whole RHDV genome, or its fragments (Asgari *et al*., 1999; Bildt *et al*., 2006; Capucci *et al*., 1998; Chrobocińska, 2007; Chrobocińska and Mizak, 2007; Farnos *et al*., 2007; Fitzner *et al.*, 2001, Fitzner, 2005; Fitzner and Kęsy, 2003; Forrester *at al*., 2006a; Forrester *at al*., 2006b; Gould *et al*., 1997; Hukowska-Szematowicz, 2006; Gall Le *et al*., 1998; Gall-Recule Le *et al*.; 2003; Matiz *et al.*, 2006; Milton *et al.*, 1992; Moss *et al*., 2002; McIntosh *et al*., 2007, Nowotny *et al.*, 1997; Niedźwiedzka-Rystwej *et al*., 2009; Pawlikowska *et al*., 2009; Rasschaert *et al.*, 1994; Schirrmeier *et al.*, 1999; Tian *et al*., 2007). The variability of nucleotide sequences within fragment of the p30 protein coding gene for the Czech and German strain pairs observed in this study ranged from 2.1% (CAMPV-562 and CAMPV-558) to 13.5% (Rossi, CAMPV-562 and CAMPV-558) and was higher compared to Polish strains SGM, KGM, PD, LUB, BLA, GSK, ŻD (1–8%) (Fitzner and Kęsy, 2003; Fitzner, 2005), and French strains (8.7%) (Gall Le *et al*., 1998). Lower variability of nucleotide sequences (0.1–9.0%) was noted within the VP60 capsid protein coding gene (or its fragments) in RHDV strains originating from various parts of the world (Hukowska-Szematowicz, 2006; Pawlikowska *et al*., 2009). Considering the facts mentioned above it is reasonable to suppose that p30 non-structural protein coding region within the RHD virus genome shows higher variability compared to the VP60 structural protein coding region.

Distance matrices presented here (Table II) show a clear genetic distance between strains originating from Europe and the USA. That phenomenon may be explained by the fact that viruses come from various continents and are separated in time. Distance matrices presented here and constructed according to the observed divergence and maximum likelihood methods proved to be reliable method of biological data interpretation. It should be noted that slightly higher matrix values were obtained when the observed maximum likelihood was used, which is consistent with assumption of the method basing on evaluation of phylogenetic distance taking into account mutation level evaluated from sequence comparison and determined by differences in sequences. And the observed divergence method is based on unchanged values obtained directly from sequence comparison by calculation of divergence coefficient for each pair of sequences (Hukowska-Szematowicz, 2006). On the other hand, the homology matrix (Table III) showed that European strains from 1980s tend to be more homologous to each other than to strains originating from other continents.

Molecular phylogenetic analysis allowed separation of the tested strains into 4 genetic groups. It is impossible to indicate a criterion based on which the strains formed those genogroups. The results of studies (Bildt *et al*., 2006; Chrobocińska, 2007; Chrobocińska and Mizak, 2007; Farnos *et al*., 2007; Fitzner *et al.*, 2001, Fitzner, 2005; Fitzner and Kęsy, 2003; Forrester *at al*., 2006a; Forrester *at al*., 2006b; Forrester *et al.*, 2003; Hukowska-Szematowicz, 2006; Gall Le *et al*., 1998; Gall-Recule Le *et al*., 2003; Matiz *et al.*, 2006; Moss *et al*., 2002; McIntosh *et al.*, 2007, Nowotny *et al.*, 1997; Niedźwiedzka-Rystwej *et al*., 2009; Pawlikowska *et al*., 2009) on the phylogenesis of RHDV strains indicate that strains form groups depending on the time of their identification or geographic region. Strain groups having a common identification time, regardless of their geographic origin, are referred to as “clusters”. In the case of G1 and G3 it is possible to indicate the existence of those clusters, and strain composition in the G4 genogroup may be interpreted by both their origin and identification time.

Evaluation of the genetic variability of the Czech and German RHD virus strains performed on a fragment of the p30 protein coding gene showed that tested strains are more variable compared to the Polish and French ones. A larger distance exists between

Czech and German strains and American ones, and the smallest between them and the European strains. Strain division into groups was associated with their identification time rather than geographic region. In 2008, RHD virus variability via recombination was described (Abrantes *et al.*, 2008; Forrester *et al.*, 2008), which will facilitate analysis of variability mechanisms of other RNA viruses.

Acknowledgements

This work was supported by grant No N308 03832/3662.

Literature

Abrantes J., P.J. Esteves and van der W. Loo. 2008. Evidence for recombination in the major capsid gene VP60 of the rabbit haemorrhagic disease virus (RHDV). *Arch. Virol.* 153: 329–335.

Asgari S., J-R-E. Hardy and B-D. Cooke. 1999. Sequence analysis of rabbit haemorrhagic disease virus (RHDV) in Australia: alterations after its release. *Arch. Virol.* 144: 135–145.

Baxevanis A.D. and B.F.F. Ouellette. 2004. Bioinformatics: a practical guide to the analysis of genes and proteins (in Polish). PWN Press, Warszawa.

Bildt M.W.G., G.H. Bolhuis, F. Zijderveld, D. Riel, J.M. Drees, A.D.M.E. Osterhaus and T. Kuiken. 2006. Confirmation and phylogenetic analysis of rabbit hemorrhagic disease virus in free-living rabbits from the Netherlands. *J. Wildlife Disease* 42: 808–812.

Capucci L., F. Fallacara, S. Grazioli, A. Lavazza, M. Lodovica Pacciarini and E. Brocchi. 1998. A further step in the evolution of rabbit hemorrhagic disease virus: the appearance of the first consistent antigenic variant. *Virus Res.* 58: 115–126.

Chrobocińska M. 2007. The phenotypic and genetic characteristic of national strains of European brown hare virus (EBHS) and rabbit haemorrhagic disease virus (RHDV) (in Polish). Habilitation dissertation. PIW-PIB, Puławy. Poland

Chrobocińska M. and B. Mizak. 2007. Phylogenetic analysis of partial capsid protein gene of rabbit haemorrhagic disease virus (RHDV) strains isolated between 1993 and 2005 in Poland. *Bull. Vet. Inst. Pulawy* 51: 189–197.

Farnos O., D. Rodriguez, O. Valdes, M. Chiong, F. Parra, J.R. Toledo, E. Fernandez, R. Lleonart and M. Juarez. 2007. Molecular and antigenic characterization of rabbit hemorrhagic disease virus isolated in Cuba indicates a distinct antigenic subtype. *Arch. Virol.* 152: 1215–1221.

Fitzner A. 2005. Habilitation dissertation. Molecular characteristic of the RHD virus strains on various phenotype (in Polish). PIW-PIB Puławy. Poland

Fitzner A. and A. Kęsy. 2003. Variability of Polish isolates of the RHD virus. (in Polish). *Medycyna Wet.* 59: 905–908.

Fitzner A., A. Kęsy, W. Niedbalski and G. Paprocka. 2001. Characterizing RHDV strains by restriction analysis of RNA (in Polish). *Medyczna Wet.* 57: 890–893.

Fitzner A., A. Kęsy, W. Niedbalski, G. Paprocka and B. Walkowiak. 1996. The identification of polypeptide VP60 national isolates of the RHD virus (in Polish). *Medycyna Wet.* 52: 303–305.

Forrester N.L., B. Boag, S. Moss, S.L. Turner, R.C. Trout, P.J. White, P.J. Hudson and E.A. Gould. 2003. Long-term survival of New Zeland rabbit haemorrhagic disease virus RNA in wild rabbits, revealed by RT-PCR and phylogenetic analysis. *J. Gen. Virol.* 84: 3079–3086.

Forrester N.L., M.I. Abubakr, E.M.E. Abu Elzein, A.I. al-Afaleq, F.M.T. Housawi, S.R. Moss, S.L. Turner and E.A. Gould. 2006a. Phylogenetic analysis of rabbit haemorrhagic disease virus strains from the Arabian Penisula: Did RHDV emerge simultaneously in Europe and Asia? *Virology* 344: 277–282.

Forrester N.L., S.R. Moss, S.L. Turner, H. Schirrmeier and E.A. Gould. 2008. Recombination in rabbit haemorrhagic disease virus: Possible impact on evolution and epidemiology. *Virology* 376: 390–396.

Forrester N.L., R. Trout, S.L. Turner, D. Kelly, B. Boag, S. Moss and E.A. Gould. 2006b. Untravelling the paradox of rabbit haemorrhagic disease virus emergence, using phylogenetic analysis; possible implications for rabbit conservation strategies. *Biol. Conserv.* 131: 296–306.

Gall Le G., C. Arnauld, E. Boilletot, J.P. Morisse and D. Rasschaert. 1998. Molecular epidemiology of rabbit haemorrhagic disease virus outbreaks in France during 1988 to 1995. *J. Gen. Virol.* 79: 11–16.

Gall-Recule Le G., F. Zwingelstein, S. Laurent, C. De Boissëson, Y. Portejoie and D. Rasschaert. 2003. Phylogenetic analysis of rabbit haemorrhagic disease virus in France between 1993–2000, and characterization of RHDV antigenic variants. *Arch. Virol.* 148: 65–81.

GeneBank, National Center of Biotechnology Information, Pub Med (www.ncbi.nlm.nih.gov) (date of the last check 6.06. 2009).

Gould A.R., J.A. Kottenbelt, C. Lenghaus, C. Morissy, T. Chalmberlain, B.J. Collins and H.A. Westbury. 1997. The complete nucleotide sequencing of rabbit haemorrhagic disease virus (Czech starain V351): use of the polymerase chain reaction to detected replication in Australian vertebrates and analysis of viral population sequence variation. *Virus Res.* 47: 7–17.

Guittre C., N. Rouen-Clouet, L. Barrakud, Y. Cherel, I. Baginski, M. Prave, J.P. Gariere, C. Trepo and L. Cora. 1996. Early stages of rabbit haemorrhagic disease virus infection monitored by polymerase chain reaction. *J. Vet. Med. B* 43: 109–118.

Hukowska-Szematowicz B. 2006. Immunological and genetical characterization of selected strains of RHD (rabbit haemorrhagic disease) virus (in Polish). Doctoral thesis. University of Szczecin, Szczecin. Poland.

Liu S.J., H.P. Xue, B.Q. Pu and N.H. Quian. 1984. A new viral disease in rabbits. Anim. Husb. *Vet. Med.* 16: 253–255.

Matiz K., K. Ursu, S. Kecskemeti, E. Bajmocy and I. Kiss. 2006. Phylogenetic analysis of rabbit haemorrhagic disease virus (RHDV) strains isolated between 1988 and 2003 in eastern Hungary. *Arch. Virol*. 151: 1659–1666.

McIntosh M.T., S.C. Behan, F.M. Mohamed, Z. Lu, K.E. Moran, T.G. Burrage, J.G. Neilan, G.B. Ward, G. Botti, L. Capucci and S.A. Metwally. 2007. A pandemic strain of calicivirus threatens rabbit industries in the Americas. *Virol. J.* 4: 96–109.

Meyers G., C. Wirblich and H.J. Thiel. 1991. Rabbit haemorrhagic disease virus-molecular cloning and nucleotide sequencing of a calicivirus genome. *Virology* 184: 664–676.

Meyers G., Ch. Wirblich, H-J. Thiel and J-O. Thumfatr. 2000. Rabbit haemorrhagic disease virus: Genome organization and polyprotein processing of a calicivirus studied after transient expression of cDNA constructs. *Virology* 276: 349–363.

Milton I-D., R. Vlasak, N. Nowotny, L. Rodak and M.J. Carter. 1992. Genomic 3' terminal sequence comparision of three isolates of rabbit haemorrhagic disease virus. *FEMS Microbiol. Letters* 93: 37–42.

Moss S.R., S.L. Terner, R.C. Trout, P.J. White, P.J. Hudson, A. Desai, M. Armesto, N.L. Forrester and E. Gould. 2002. Molecular epidemiology of rabbit haemorrhagic disease virus. *J. Gen. Virol*. 83: 2461–2467.

Niedźwiedzka-Rystwej P., M. Pawlikowska, B. Hukowska-Szematowicz, B. Tokarz-Deptuła and W. Deptuła. 2009. Immunological and genetic studies of RHD (rabbit haemorrhagic disease) virus strains. *Centr. Eur. J. Immunol.* 34: 61–67.

Nowotny N., C.R. Bascunana, A. Ballagi-Pordany, D. Gavier-Widen, M. Uhlen and S. Belak. 1997. Phylogenetic analysis of rabbit haemorrhagic disease and European brown hare syndrome viruses by comparison of sequences from the capsid protein gene. *Arch. Virol.* 142: 657–673.

Pawlikowska M., B. Hukowska-Szematowicz and W. Deptuła. 2009. Genetical characteristic of six strains of RHD (rabbit haemorrhagic disease) virus orginating from Europe. *Centr. Eur. J. Immunol.* 34: 7–13.

Rasschaert D., S. Huguet, M.F. Madelaine and J.F. Vautherot. 1994. Sequence and genomic organization of a rabbit haemorrhagic disease virus isolated from a wild rabbit. *Virus Genes* 9: 121–132.

Schirrmeier H., I. Reimann, B. Kollner and H. Granzow. 1999. Pathogenic, antigenic and molecular properties of rabbit haemorrhagic disease virus (RHDV) isolated from vaccinated rabbits: detection and characterization of antigenic variants. *Arch. Virol.* 144: 719–735.

Tian L., J. Liao, J. Li, W. Zhou, X. Zhang and H. Wang. 2007. Isolation and identification of non-haemagglutinating strain of rabbit hemorrhagic disease virus from China and sequence analysis fort he VP60 gene. *Virus Genes* 35: 745–752.

Wirblich Ch., H-J. Thiel and G. Meyers. 1996. Genetic map of the calicivirus rabbit haemorrhagic disease as deduced from *in vitro* translation studies. *J. Virol.* 70: 7974–7983.