Original article

Identification of Polish RHDVa subtype strains based on the analysis of a highly variable part of VP60 gene

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Abstract

In order to determine the genetic variability of Polish RHD virus strains and to confirm the presence of genetic variant (RHDVa) subtype the partial nucleotide sequences of capsid protein gene, including two highly variable regions C and E, were examined. Phylogenetic analyses of 15 viral strains obtained over 18 years revealed the presence of three genetic groups. The oldest RHDV strains exhibit very close amino acid sequence similarity (98-99%) to the German FRG89 reference strain and most of European strains of the same period, as well as Chinese isolate from 1984. The HA-negative strains and isolates with variable reactivity in the HA test belong to the second subgroup and exhibit an intermediate level of variability (about 3%) in the analysed VP60 gene fragment. The most genetically variable strains (6-7%) clustered to RHDVa subtype. The analysis of nucleotides and amino acid sequences demonstrated three pairs of well conserved RHDV strains, isolated over 3, 6 and 10-year period.

Key words: RHD, VP60, sequence analysis, phylogenetic analysis, RHDVa variant

Introduction

A deadly viral plague of rabbits has been known for over 25 years. Rabbit haemorrhagic disease, which seriously threatened the global development of rabbit breeding and meat production, originated in China in 1984 (Liu et al. 1984). Over the few years the plague spread rapidly throughout the world affecting many countries, mainly in Asia and Europe. The outbreaks of RHD were noted in North Africa, in Mexico, USA and Cuba on American continent. Due to the extremely high morbidity and mortality rate reaching up to 100%, in the late 90s of the 20th century, the disease was introduced in the experimental condition into New Zealand and Australia, as biological weapon, in order to reduce the number of wild rabbits (Liu et al. 1984, Gregg et al. 1991, Morisse et al. 1991, Cooke 2002, Oem et al. 2009). The only known species of animals susceptible to the disease are wild and domestic rabbits belonging to the *Oryctolagus cuniculus*. The disease was a major cause of death of animals aged over 8 weeks, while the young animals were immune (Gregg et al. 1991, Gould et al. 1997). The etiological agent – rabbit heamorrhagic disease

virus (RHDV) is classified in the family *Caliciviridae*. The virion with diameter 28-40 nm contains a single-stranded 7.5 kb RNA of positive polarity encoding both non-structural and structural proteins in one ORF1. The virus capsid is non-enveloped and consists of 180 copies of structural protein VP60 (Parra et al. 1990, Meyers et al. 1991, Ohlinger et al. 1991).

Although the RNA viruses are characterised by the great potential of variability, in the initial period of RHD spreading the isolated strains were conservative in terms of antigenic and genetic features regardless of time and place of geographical occurrence. Amino acid sequence comparison of Australian RHDV strains demonstrated no more than 2% variability after 2 years circulation in the environment (Gould et al. 1997, Novotny et al. 1997, Le Gall et al. 1998, Asgari et al. 1999). The first significant changes in the properties of the virus were related to the lack of haemagglutination capacity of some strains (Chasey et al. 1995, Kesy et al. 1996). In 1996 the occurrence of antigenic and genetic variant RHDVa strains were reported in Italy and Germany (Capucci et al. 1998, Schirrmeier et al. 1999). In the same time non-pathogenic calicivirus (RCV) was also detected in rabbits, characterized by genetic relatedness to the RHD virus and a different tropism for the gastrointestinal tract (Capucci et al. 1998). The occurrence of non-pathogenic strains of RCV type responsible for persistent infections, or asymptomatic infections in wild rabbits have been confirmed recently in Australia and France. It has been shown that these caliciviruses can induce antibodies reacting specifically with RHD virus and provide partial protection against lethal infection with RHDV (Strive et al. 2009, 2010, Le Gall-Reculé et al. 2011).

In Poland, the first cases of the highly contagious and lethal disease of rabbits were confirmed in the spring of 1988 (Gorski et. al 1988). The disease has spread very rapidly during following years, encompassing majority of the country and contributed to the enormous losses in rabbittries, regardless of race and type of breeding rabbits (eg. NZ, mixed-race, giant or miniatura). In 1994 non-haemagglutinating BLA strain – a phenotypic variant of RHD was isolated from an outbreak in a small rabittry. RHD viral antigen was detected by ELISA and confirmed by PCR (Kesy et al. 1996, Fitzner et al. 2003). For the first time Polish RHDV strains exhibiting features similar to antigenic and genetic variant RHDVa have been isolated in the middle of the first decade of 21st century (Chrobocińska et al. 2007, Fitzner 2009).

The aim of the present study was the molecular characterisation of the recently described RHDV strains exhibiting the differences in antigenic and hae-magglutinating reactivity. The assessment of genetic variability of virus strains isolated in Poland since 1988 and their phylogeny was performed. In order to confirm the presence of genetic variant (RHDVa) and to determine the genetic lineage of Polish RHDV strains, the partial nucleotides sequences of VP60 gene, including two highly variable regions C and E, were examined.

Materials and Methods

RHDV. A total of 16 RHDV strains collected from dead domestic rabbits, representing 15 different Polish RHD outbreaks and one external V351 Czech strain were included in the study. The analysis involved eight recently described strains from the years 2003-2004 (CB, GRZ, KRY, LBN, OPO, ROK, ZDU, ZKA), strain DCE obtained in 2006, five strains originated between 1988 and 2000 (KGM, BLA, MAL, GSK, ZD0) and passage of isolate V351 from 2000 (kindly provided from the collection of Professor Deptula, University of Szczecin, Poland). The virus strains prepared as 20% liver homogenates were conserved with glycerol (1:1) then frozen and stored at -18 -26°C or mixed with preservative (15% v/v), freeze-dried and stored at +5°C (±3). To study the phylogeny the following nucleotide sequences of 18 RHDV reference strains obtained from GeneBank were used. FRG (M67473), Bs89 (X87607), AST-89 (Z49271), RHDV-SD (Z29514), Pv97 (EU250330), Vt97 (EU250331), Iowa (AF258618), whn-1 (DQ069280), WX-China 1984 (AF402614), Rainham (AJ006019), Cub5-04 (DQ841708), Hartmannsdorf (EF558586), Triptis (Y15442), Frankfurt (Y15424), Hagenow (Y15441), 99-05 (AJ302016), 00-Reu (AJ303106), RCV (X96868).

Haemagglutination assay (HA). The assay was carried out in U-bottomed microtitration plate. Serial two fold dilution of the viral suspension samples, starting from 1:10 were performed in a volume of 100 μl and incubated with equal volume of 0.75% human red blood cells suspension. The haemagglutination was assayed in 0.85% NaCl (pH 7.5) at room temperature (18-26°C) and PBS (pH 6.5) at +5°C (±3°C). The results were read visually after 1.5-2 h of incubation.

RT-PCR. Total RNA was extracted from liver homogenates using the RNeasy Mini Kit (Qiagen Inc.) and eluted in 35 μl H2O. RNA was incubated for 15 min in RT with reaction mixture. cDNA synthesis was performed at 42° for 60 min in a 60 μl reaction using 1 μl oligo dt15 (Promega) or RHDV-specific antysense P5(-) 7071-7051 primer (5'GCAC-CTGCAAGTCCCAATCCG), PCR nucleotide mix

and AMV reverse transcription enzyme (Promega). Three fragments of VP60 gene were amplified by PCR method, using the following sequences of primers: P1(+) 6069 (5'CAGGTGGAAC-GGCCAAATAG), P2(-) 6654 (5'AGGAG-TGCCGGGTGTGGTTAC, P3(+) 6300 (5'CCAGACGGCTTTCCTGACATG), P4(-) 6894 (5'CACACTTAAACCAATCTCCAT based on published sequence of German and Italian RHDV strains (Capucci et al. 1996, 1998). The primers and sequencing were done by Institute of Biochemistry and Biophysics (IBB, Warsaw).

RFLP. The amplicons of 825 bp (P1-P4) of ten isolates were digested with Eco147 enzyme (Fermentas) using restriction site within E region of capsid protein gene. PCR and RFLP products were analysed on a 1,5% agarose gel, stained with ethidium bromide and visualised with ImageStore documentation system (UVP).

Sequencing and phylogenetic analysis. A total of 32 PCR products that covered regions C and E of VP60 gene were gel-extracted using a QIAquick Gel Extraction Kit (Qiagen) and directly sequenced in both orientations using ABI Prism BigDye™ terminator v3.1 Cycle sequencing kit and ABI37730x1 DNA sequencer (Applied Biosystems). Based upon two overlapping fragments (P1-P2, P3-P4) a 262 amino acid sequence of 16 strains was reviewed manualy. For viral genome alignments sequences of known RHDV strains from GeneBank were generated using the BLASTn (Altschul et al. 1990). The phylogenetic tree was constructed from 530 bp (P1-P2) fragment using neighbour-joining method with 1000 bootstraps values (Tamura et al. 2007).

Results

HA test was performed under two different conditions of temperature and pH after the longest storage period for confirmation of haemagglutination activity and evaluation of stability of HA titer of RHDV strains. Generally, the HA test results confirmed the significant haemagglutinating activity of most of RHDV strains. It has been shown that HA titer of virus strains KGM, MAL, POZ, ZD0 and the group of six strains characterized as RHDVa did not change when compared to the initial titer in the entire long-term storage, both in terms of incubation at room temperature at pH 7.5 buffer as well as at +5°C, pH 6.5 buffer (Table 1).

Virus strains, stored in frozen suspension or in lyophilized form at +5° (±3) remained active without loss of HA titer for a very long time. The maximum HA titers in the range of 10,240 to 20,480 were re-

Table 1. Stability of haemagglutination titer of RHDV strains.

RHDV strain	Storage		HA titer	
	form	time (month)	pH 6.5/5°C	pH 7.5 /18-26°C
ROK	susp.	75	10240	5120
GRZ	susp.	74	20480	20480
CB	susp.	67	10240	5120
ZKA (L4)	susp.	67	20480	20480
DCE (L1)	susp.	21	1280	10
DCE (L2)	susp.	21	2560	160
DCE (L3)	susp.	21	1280	40
KRY	susp.	71	10240	10240
ZDU (L1-L6)	susp.	90	10240	5120
LBN	susp.	74	2560	160
OPO	susp.	74	negative	negative
GSK	f-d	146	5120	80
POZ	f-d	134	2560	320
ZD0	susp.	132	2560	640
MAL	f-d	192	1280	5120
BLA	f-d	192	negative	negative
KGM	susp.	54	5120	5120
V-351	susp.	126	2560	5120

Susp. (suspension) / f-d (freeze-dried); L – liver (number of rabbit).

corded for strains GRZ and ZKA, respectively. Both strains, HA-negative BLA and OPO, failed to agglutinate, and as it was previously demonstrated, the lack of haemagglutinaton persisted even after the double passage in rabbits. However, for some virus samples a gradual decrease of HA titer was reported when tested in a buffer at pH 7.5. The reduction of HA activity was observed for LBN, DCE and GSK strains. Further investigation of isolate LBN showed a significant decrease of initial haemagglutination titer for other suspensions of the same liver, shortly after homogenate was prepared.

The genetic material of 16 RHDV strains was amplified by the RT-PCR method. Two sets of 16 PCR products of approximately 585 bp for P1-P2 and P3-P4 fragments and 16 amplicons of 825 bp of VP60 gene were obtained. RFLP analysis of 825 bp fragment detected no alterations in the Eco147 recognition sequence site localised in the stabile part of the E variable region of capsid protein gene. (Fig. 1). Among the 10 analysed strains, all presented the same restriction pattern with two bands of 470 bp and 360 bp. Nucleotide sequence analysis confirmed the results obtained by RFLP – the stability of strains in this
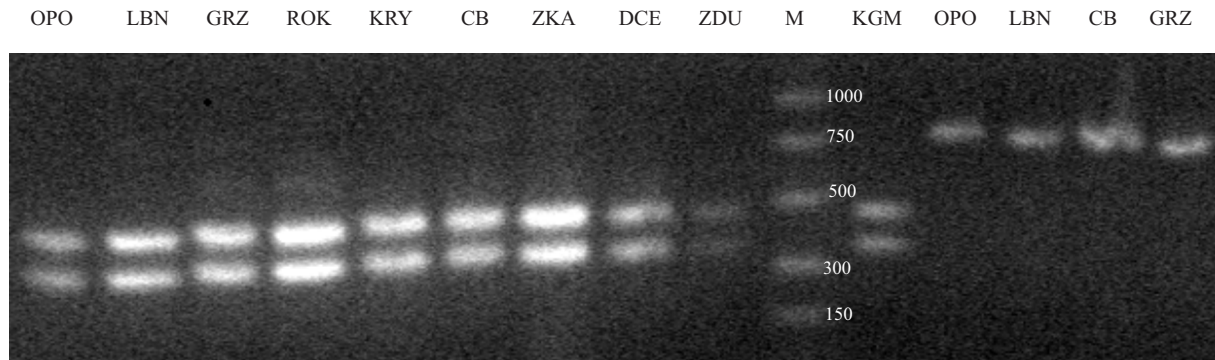
Fig. 1. RFLP and PCR analysis of capsid protein gene of RHDV strains in 1,5% agarose gel. From left – a 825 bp fragment of nine RHDV strains digested with Eco147: OPO, LBN, GRZ, ROK, KRY, CB, ZKA, DCE, ZDU, Molecular weight marker (Promega) 1000, 750, 500, 300, 150, 50 bp, KGM, a 825 bp PCR products of four RHDV isolates: OPO, LBN, CB, GRZ.
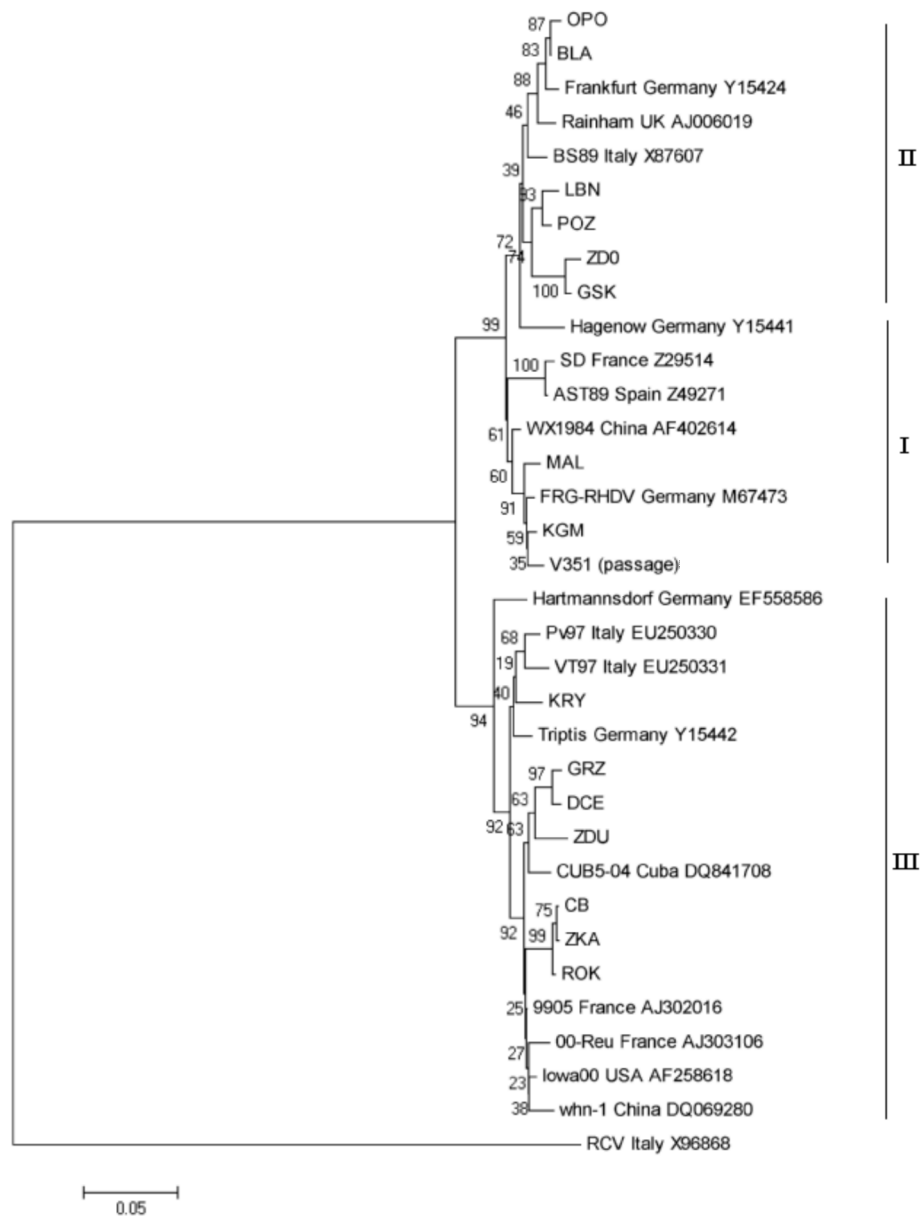
Fig. 2. Phylogenetic tree of 34 RHDV isolates. Analysis made by MEGA software, using neighbour-joining method with bootstrap values calculated for 1000 replicates.

part of the hypervariable region E of VP60 gene was found. The dendrogram of 34 RHDV strains reveals the presence of three separate genetic groups (Fig. 2). Sequences of Polish RHDV strains are located in each of these genogroups. The first (I) includes the oldest RHDV strains isolated over the first decade after disease was discovered. The isolates KGM (1988) and MAL (1994) clustered with classical RHDV: Chinese isolate of 1984, the German reference strain FRG89 (M67473), French SD and Spanish AST both isolated from wild rabbits in 1989. To the second group (II), represented by Italian strain Bs89, belonged two isolates, OPO and LBN detected in 2004, GSK originated from miniatura rabbits (1998), POZ (1999), ZD0 (2000), and oldest of them HA-negative BLA (1994). A common feature of many of these strains is the lack of haemagglutination or a variable profile of haemaglutination (Hagenow, LBN). It was shown that the HA-negative strains, BLA and OPO and other strains with these properties (Frankurt and Rainham), were derived from the same branch of the phylogenetic tree. The strains with variable properties of haemagglutination: GSK, LBN also form a separate cluster together with Hagenow isolate. Analysis of the hypothetical, discontinuous domain responsible for haemagglutination (Capucci et al. 1998) showed mutation at amino acid position 481 similarly to Italian Bg97 and German Frankfurt HA-negative strains but no alterations at positions 305, 399, 476 in two non-haemagglutinating Polish strains BLA and OPO. The amino acid 399 confirmed the change from T to I in strains GSK and ZD0, similarly as in strain Pv97. As a part of this domain we suggest also an amino acid position 506, present in a strain BLA and Frankfurt.

The third genetic group (III) refers to the strains identified as an antigenic and genetic variant RHDVa and includes seven Polish isolates (CB, DCE, GRZ, KRY, ROK, ZDU, ZKA). The main representatives of this group are the Italian isolates Pv and Vt isolated in 1997, German Hartmannsdorf and Triptis (1996), U.S. Iowa (2000), Cuban Cub5-04 (2004) and Chinese whn-1 (2005). The 786 bp fragment of VP60 capsid protein gene from 16 evaluated isolates was obtained. The sequences of deduced 262 amino acids fragment of these isolates and 17 RHDV sequences from Gene Bank were compared to reference strain FRG89 (M67437) (Fig. 3). The overall homology of analysed fragment of capsid protein gene encompassing two variable regions C and E ranged from 93.5 to 99% of amino acids. The most variable strains (6-7% amino acid differences) are represented by seven isolates which, in the previous work based on the antigenic reactivity in ELISA, were classified as RHDVa (Fitzner 2009). The lowest variation (0.8-1.5% amino acid

differences as compared to FRG89) was found in KGM, MAL, and V-351 isolates. Amino acid sequence obtained for passage V351 was more similar to the original partial sequence (Z11535), with same amino acid mutation at position 480, than to the complete genome sequence (U54983). The intermediate group with the variability of about 3% of amino acids includes four strains GSK, POZ, ZD0, LBN with haemagglutinating activity as well as two non-haemagglutinating phenotypic variants BLA (1994) and OPO (2004). The isolates GRZ, CB, ZKA, DCE, ROK, KRY, ZDU exhibited a considerably higher variability within regions C and E of VP60 gene than other analysed strains.

Compared to FRG89, the highest variability of these strains was detected in the region C (20%-30%), initial (EI) and final (EII) part of region E (26% and 10% amino acid differences). The highest number of amino acids changes were detected within region C and F in the strain KRY, and within region E of DCE isolate. It was found that the distribution of amino acid changes within the first part of region E was identical in all groups. Sequence comparison of analysed strains showed their similarity to the Italian and German isolates known as antigenic and genetic RHDV variant – RHDVa. Variability between the other tested isolates and FRG reference strain within the regions C and E was significantly lower and did not exceed 10% and 6%, respectively. No amino acids changes were found within region E between strains FRG and KGM, MAL, BLA, OPO. KGM was one of the first two strains of RHD virus detected in Poland in 1988, while BLA was the first recognized non-haemagglutinating phenotype variant (Gorski et al. 1988, Kesy et al. 1996). Also no changes within EI region for strains GSK, POZ, ZD0, LBN were found.

Discussion

The previous studies on the Polish RHDV strains isolated from 1988 to 2000 identified two genogroups related to German strain FRG89 and Italian BS89. Analysis of two fragments of genome encoding a non-structural part and 5' conserved fragment of capsid protein gene indicated two possible ways of virus introduction to Poland (Fitzner et al. 2003). Actually, three separate genetic groups of RHDV comprising classical RHDV, antigenic and genetic variant RHDVa, and the intermediated group which combines classical strains and phenotypic variant, were demonstrated. The results of sequence analysis confirmed that seven out of nine analysed strains detected between 2003 and 2006 share the antigenic and genetic features of RHDVa subtype. Two other

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                   ...............B.............................C........D....

FRG89    262    VGLQPVPGGFSTCNRHWNLNGSTYGWSSPRFGDIDHRRGSASYSGSNATNVLQFWYANAG   321
KGM             ................................A..........P...............
MAL             ................................A..........P...............
V-351           ................................A.....K....T...............
BLA             ................................A..........P.N.............
OPO             ................................A..........P.N.............
GSK             ................................A..........P...............
ZD0             ................................A..........P...............
POZ             ................................A.....S.....P.N.............
LBN             ................................A.....S.....P.N.............
GRZ             ................................A...........N.S............
CB              ................................A...........N.S............
ZKA             ................................A...........N.S............
ROK             ................................A...........N.S............
DCE             ................................A...........N.S............
KRY             ................................A...........NTS............
ZDW             ................................A.............S............
Rainham         ................................A..........P.N.............
Frankfurt       ................................A..........P.N.............
Hagenow         ................................A..........P...............
Hartm.          .........................................P.N.S............
Triptis         .........VF.....................A...........N.S............
Bs89            ................................A..........P...............
Pv97            ................................A..........A.N.S............
Vt97            ................................A...........N.S............
Iowa            ................................A...........N.S............
CUB5            M...............................A...........N.S............
whn-1           ................................A.........FA.N.S............
00-Reunion      ................................A.............S............
99-05           ................................A.............S............
SD89            ................................A..........P.N.............
AST89           ................................A..........P.N.............
WXChina84       ................................A..........P...............
RCV             ........L.....................T.......ASQPG.N---...........   318


                   ........D...............E(I)............................

FRG89    322    SAIDNPISQVAPDGFPDMSFVPFNGPGIPAAGWVGFGAIWNSNSGAPNVTTVQAYELGFA   381
KGM             ...........................................................
MAL             ...........................................................
V-351           ...........................................................
BLA             ..V........................................................
OPO             ..V........................................................
GSK             ..V........................................................
ZD0             ..V........................................................
POZ             ...........................................................
LBN             ...........................................................
GRZ             ........................S.N..T......G.....N...AA...........
CB              ........................S.N..T......G.....N...AA...........
ZKA             ........................S.N..T......G.....N...AA...........
ROK             ........................S.N..T......G.....N...AA...........
DCE             ........................S.N..T......G.....N...AA...........
KRY             ........................S.N..T......G.....N...AA...........
ZDW             ........................S.N..T......G.....N...AA...........
Rainham         ..V........................................................
Frankfurt       ..V........................................................
Hagenow         ..................................................R...D
Hartm.          ..........................N..T......G.....N...AA...........
Triptis         ........................S.N..T......G.....N...AA...........
Bs89            ..V........................................................
Pv97            .......................S.NV.T......G.....N...AA...........
Vt97            .......................S.NV.T......G.....N...AA...........
Iowa            ........................S.N..T......G.....N...AA...........
CUB5-04         ........................S.N..T......G.....N...AA...........
whn-1           ........................S.N..T......G.....N...AA...........
00-Reunion      ........................S.S..T......G.....N...AA...........
99-05           ........................S.N..T......G.....N...AA...........
SD89            ...........................................................
AST89           ...........................................................
WXChina84       ...........................................................
RCV             ..V....C.............L...NV.T..............................   378
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Fig. 3.

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                              ............................E(II)......................F..
        FRG89  382  TGAPGNLQPTTNTSGAQTVAKSIYAVVTGTAQNPAGLFVMASGIISTPNASAITYTPQPD  441
        KGM         ...........................................................
        MAL         ...........................................................
        V-351       ...........................................................
        BLA         .................................................N.........
        OPO         .................................................N.........
        GSK         ................I...........G.....................N.........
        ZD0         ................I...........G.....................N.........
        POZ         ...........................G.....................N.........
        LBN         ...........................G.....................N.........
        GRZ         ....N.......................N...T........V.......V.........
        CB          ....N.......................N...T........V.......V.........
        ZKA         ....N.......................N...T........V.......V.........
        ROK         ....N.......................N...T........V.......V.........
        DCE         ....N.......................N...T........V.....N.V.........
        KRY         ...........................G.....................N.........
        ZDW         ....N.......................N...T........V.......V.........
        Rainham     .................................................N.........
        Frankfurt   ........................................V........N.........
        Hagenow     ....S...................................V........N....A....
        Hartm.      ....N.......................N...T........V.......V.........
        Triptis     ....N.......................N...T........V.......V.........
        Bs89        ........................................V........N.........
        Pv97        ....N...........I...........N...T........V.......V.........
        Vt97        ....N.......................N...T........V.......V.........
        Iowa        ....N.......................N...T........V.......V.........
        CUB5-04     ....N.P.....................N...T........V.......V.........
        whn-1       ....N.......................N...T........V.....N.V.........
        00-Reunion  ....N.......................N...T........V.....N.V.........
        99-05       ....N.......................N...T........V.......V.........
        SD89        .............S..........................V....S.N.........
        AST89       .............S..........................V....S.N.........
        WXChina84   ........................................V..................
        RCV         ....N....A.....S.I........S..AN.................T.R.........  438


                              ..........................F.................................
        FRG89  442  RIVTTPGTPAAAPVGKNTPIMFASVVRRTGDVNATAGSANGTQYGTGSQPLPVTIGLSLN  501
        KGM         ...........................................................
        MAL         ...................................V.......................
        V-351       ....................................V......................
        BLA         ..................................S.......................
        OPO         ..................................T...S...................
        GSK         ...................................T......................
        ZD0         ...A...............................T......................
        POZ         ...................................T......................
        LBN         ...................................T......................
        GRZ         .................................A...T....................
        CB          .................................A..T.....................
        ZKA         .................................A...T....................
        ROK         .................................A...T....................
        DCE         ....A..............................T......................
        KRY         ..........T......................A...T....................
        ZDW         .................................A...T....................
        Rainham     .................................S.......................
        Frankfurt   .....................................S...................
        Hagenow     ...................................V......................
        Hartm.      ...........................................................
        Triptis     .................................A...T....................
        Bs89        ...........................................................
        Pv97        ...........................................................
        Vt97        .................................A.......................
        Iowa        .................................A...T....................
        CUB5-04     ....................P.........A...T....................
        whn-1       ........................K......A...T....................
        00-Reunion  .................................A...T....................
        99-05       .................................A...T....................
        SD89        .....................................R...................
        AST89       ...........................................................
        WXChina     ...........................................................
        RCV         ...NA...........V...............E...D....................  498
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Fig. 3.

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                      .........F............
FRG89   502   NYSSALMPGQFFVWQLTFASGF   523
KGM           .....................
MAL           .....................
V-351         .....................
BLA           ....S................
OPO           .....................
GSK           .....................
ZD0           .....................
POZ           .....................
LBN           .....................
GRZ           .....................
CB            .....................
ZKA           .....................
ROK           .....................
DCE           .....................
KRY           .....................
ZDW           .....................
Rainham       .....................
Frankfurt     ....S................
Hagenow       .....................
Hartm.        ......L..............
Triptis       ......V..............
Bs89          .....................
Pv97          ........              509
Vt97          ........              509
Iowa          .....................
CUB5-04       ......I..............
whn-1         .....................
00-Reunion    .....................
99-05         .....................
SD89          .....................
AST89         .....................
WXChina84     .....................
RCV           ......T.........N.....   520
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Fig. 3. An amino acid sequence alignment part of VP60 capsid protein gene of the 34 RHDV isolates.

strains of RHDVa were detected in Poland at the same time (Chrobocińska et al. 2007). These results point to the emergence of antigenic variant in Poland in the middle of the first decade of XXI century and are consistent with epizootic data from other European countries and continents. In the same period RHDVa strains were isolated in France, Hungary, Cuba, U.S., China, Korea and Uruguay (Le Gall-Reculé et al. 2003, Matiz et al. 2006, McIntosh et al. 2007, Tian et al. 2007, Oem et al. 2009).

Our results complement the data concerning the significant spreading of RHD outbreaks in recent years caused by RHDVa variant, an enormous dynamics of these strains and the evolution process of the virus. On the other hand, the considerable stability of the virus characteristics in regard of pathogenicity, antigenicity and immunological properties observed during the first 10-15 years of disease spreading should be mentioned. However, despite the passing years, the source of the virus has not been clearly defined. In the light of the already known results of retrospective studies and new data evidence for the presence of non-infectious viral ancestor of RHDV appear to be certain. Numerous studies indicated the presence of avirulent or benign (harmless) forms of

RHDV existing long time before the first RHD outbreak was detected. The occurrence of non-pathogenic strains of RCV type responsible for persistent infections, or asymptomatic infections, in wild rabbits have been confirmed recently in Australia and France. It was shown that these caliciviruses can induce antibodies reacting specifically with RHD virus and provide partial protection against lethal infection with RHDV (Rodak et al. 1990, Moss et al. 2002, Forrester et al. 2003, 2007, 2009, Strive et. al 2009, 2010, Le Gall-Reculé et al. 2011).

In addition to the RHDVa, our analysis demonstrated the presence of well conserved RHDV strains. Three pairs of strains BLA/OPO GSK/ZD0, POZ/LBN, that were isolated at 10, 3, and 6 year intervals, showed very close nucleotide sequence homology and differed by 2, 6 and 7 nucleotides substitutions respectively, without any amino acid mutation. Only in case of pair POZ/LBN the strains were isolated from the geographically close regions. In two other cases the place of origin was separated by 350-400 km. These observations demonstrate the ability of the virus to persist and maintain in the environment over long time. Coexistence of different genetic lineages of classic RHDV in limited area was described recently in Scotland (Forrester et al. 2009).

The presented results confirmed the occurrence in Poland of RHDVa subtype isolates sharing very close sequence homology with Italian and German RHDVa isolates, but detection of well conserved RHDV isolates similar to classical and non-haemagglutinating strains provides an evidence for the coexistence of the multiple variants of the virus in the specific ecological area. Recent emerging of an increasing number of non-pathogenic strains of RCV or non-pathogenic lagovirus (NP-LV) in the world is a reason for further studies in this field.

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