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Original article

Detection of rabbit haemorrhagic disease virus 2 (GI.2) in Poland

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Abstract

In this paper we present the first cases of rabbit haemorrhagic disease virus 2 (RHDV2 – GI.2) in Poland. The virus was detected in liver samples of RHD-suspected rabbits from Lodzkie and west Pomeranian voivodeships. In both cases, the typical clinical symptoms of the disease were observed despite the fact that the rabbits were previously vaccinated against RHD. In order to extend the analysis of the RHDV2 strain infecting the rabbits, the entire VP60 and NSP genes were amplified and sequenced. The results of rRT-PCR assay have shown that tested RHDV samples were positive for the presence of RHDV2. In the phylogenetic analysis of *vp60*gene the first Polish RHDV isolates (RED 2016 and VMS 2017) clustered together with the reference RHDV2, meaning they represent new evolutionary RHDV linkeages. The first Polish RHDV2 isolates showed about 97% nucleotide sequence identity with the reference RHDV2 strains and approximately 18% difference from classic RHDV and RHDVa variants.

Key words: rabbit, RHDV2, Poland

Introduction

Rabbit haemorrhagic disease (RHD) is a highly infectious and fatal viral infection of wild and domestic rabbits (*Oryctolagus cuniculus*), responsible for important economic losses in the rabbit industry (Abrantes et al. 2012b). The disease was first reported in 1984 in China following the importation of commercially bred Angora rabbits from Germany (Liu et al. 1984). RHD is caused by rabbit haemorrhagic disease virus (RHDV), a *Lagovirus europaeus* (GI.1) of the family *Caliciviridae* (Le Pendu et al. 2017). Phylogenetic analysis revealed six pathogenic RHDV genogroups (G1-G6) and non-pathogenic related forms (Le Gall-Reculé et al. 2003). The origin and emergence of RHDV as a pathogenic virus affecting the European rabbits is still unclear but one of the hypothesis involves a direct evolution from non-pathogenic form of the virus (Kerr et al. 2009) and is an evidence for the existence of RHDV as a non-pathogenic form before the first documented RHD outbreak (Abrantes et al. 2012b). In 1996, the occurrence of a new RHDV virus - antigenic variant RHDVa - was reported in Italy and Germany (Capucci et al. 1998, Schirrmeier et al. 1999). More recently, in 2010, a new RHDV form, originally designated as RHDV2 (also named RHDVb), genetically and antigenically different from the classic RHDV and RHDVa, emerged in domestic and wild rabbits in France

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(Le Gall-Reculé et al. 2011, Dalton et al. 2012). Besides the genetic and antigenic differences between GI.2 (RHDV2) and GI.1 (RHDV/RHDVa), according to the new nomenclature of pathogenic lagoviruses (Le Pendu et al. 2017), RHDV2 resulted in atypical RHD outbreaks that led to mortality in both vaccinated adult rabbits (Le Gall-Reculé et al. 2011, Le Gall-Reculé et al. 2013) and young rabbits (Dalton et al. 2012) that are typically resistant to RHDV. Rabbits vaccinated against RHDV are effectively protected against RHDV infection but only partially protected against RHDV2 (Le Gall-Reculé et al. 2011, Le Gall-Reculé et al. 2013). Phylogenetic analysis of full-length major capsid protein (VP60) gene showed that RHDV2 creates a novel phylogenetic group that falls between the non-pathogenic rabbit caliciviruses RCVs: GI.3 /GI.4 (i.e. non-pathogenic European RCV-E1 and the weakly pathogenic Michigan rabbit calicivirus (MRCV) (Bergin et al. 2009) and the Australian non-pathogenic RCV-A1 (Le Gall-Reculé et al. 2011, Le Pendu et al. 2017). In addition, among RHD viruses detected in Portugal, recombination processes have been identified, and as a consequence the presence of at least two types of pathogenic recombinants was described including classic RHDV G1/RHDV2 and non-pathogenic RCV/RHDV2 (Lopes et al. 2015).

Materials and Methods

Origin of the samples

Three newly detected RHDV strains that came from the domestic rabbits that died between September 2016 and June 2017 were included in the study. RHDV RED 2016 was isolated on September 2016 from 4 mixed-breed rabbits (age of 3.5 months) that died at a small-scale rural farm Reduchów near Zdunska Wola, Lodzkie voivodeship. In total, the typical clinical symptoms of the disease have been confirmed in 15 dead rabbits despite the fact that all animals (35 rabbits) were vaccinated against RHD using monovalent vaccine "Pestorin" about 1.5 month before outbreak. RHDV VMS 2017 was isolated from pet rabbit, about 2 years old, with suspected RHD, previously vaccinated using Nobivac Myxo RHD; Szczecin, west Pomeranian voivodeship. RHDV BBI 2017 RHDV was isolated from two mixed-breed rabbits reared at smallholder farm at Bialobrzegi, Mazowieckie voivodeship. The rabbits were vaccinated with Pestorin Mormyx, 15 and 40 days before death, respectively.

Control samples

RHDV, RHDVa strains isolated in Poland and two RHDV type 2 reference strains (kindly received from ANSES, Ploufragan, France), as well as healthy rabbit liver homogenate were included as positive and negative control.

Virological examination

The liver samples were homogenised in phosphate-buffered saline (PBS) to the final concentration of 20 % (w/v) and tested for the presence of RHDV antigen using various ELISA tests (CR Mab ELISA - IZSLER Brescia, Italy; Ingezim RHDV DAS - Ingenasa, Spain; in-house polyclonal ELISA (Pab) and haemagglutination assay (HA). Both commercial ELISA kits containing monoclonal antibodies allowed the detection of all known pathogenic RHDV/RHDVa and RHDV2 and were used according to the manufacturers' instructions. HA and Pab ELISA assays were used as described previously (Fitzner et al. 2012, 2014).

Molecular analysis

Total RNA was extracted from 100 μL of liver homogenate as described previously (Fitzner et al. 2012) and used to molecular tests.

The real-time reverse transcription (rRT-PCR). One TaqMan primer/probe set from VP60 gene of RHDV2 according to Duarte (Duarte et al. 2015a) was used to amplify RHDV2. The reaction mixture at the volume of 25 μL contained RHDV2 forward primer (5'TGGAACTTGGCTTGAGTGTTGA-3'), RHDV2 reverse primer (5'ACAAGCGTGCTTGTGGACGG-3'), RHDV2 probe (FAM-TGTCAGAACTTGTTGACAT CCGCCC-TAMRA). The assay was performed in MicroAmp optical 96-well reaction plate in one-step reaction using the QuantiTect Probe PCR Kit (QIAGEN). The reaction mixture at the volume of 20 μL contained: 12.5 μL of 2x QuantiTect Probe RT-PCR Master Mix, 1 μL (20 pmol) of each of the primers: RHDV2-F, RHDV2-R, 0.5 μL (5 pmol) of RHDV probe, 1.25 μL of $MgSO_4$ (25 mM), 0.1 μL of RNasin, 0.2 μL of QuantiTect RT Mix, and 4.45 μL of RNase-free water. Five microlitres of extracted RNA were added to the reaction mix (total volume 25 μL) and the plate was transferred to the thermal cycler (7300 Real Time PCR System, Applied Biosystems) and amplification was carried out using the following programme: 50°C for 45 min, one cycle (reverse transcription), 95°C for 15 min (one cycle) to activate the DNA polymerases and inactivate the reverse transcriptases, and 50 cycles at 95°C for 15 s, at 60°C for 30 s and at 72°C for 30 s. Fluorescence was measured at the end of the 60°C

Table 1. RHDV antigen detection in ELISA/HA tests and C_T values of reference and test samples obtained by rRT-PCR assay.

RHDV strain	Ingezim DAS (OD 450 nm)	CR Mab ELISA (OD 490 nm)	Pab ELISA (OD 490 nm)		HA (titer)	Real-time	
	cut-off: 0.342	(dil. 1:5)	(dil. 1:5)	% OD pos*		RHDV1 Probe	RHDV2 Probe
RED 2016	0.766	2.05	0.514	22	10240	negative	14.08
VMS 2017	0.910	1.7-1.98	0.446	19	20480	negative	13.52
BBI 2017	0.848	1.783	1.007	42.9	10240	21,73	negative
C (+) RHDV Pab	1.208	1.758	2.356	100	10240	17.5	negative
C (+) RHDVa	0.556	1.671	1.238	52.7	20480	17.5	negative
C (+) RHDV HA-neg	1.428	1.818	2.3	97.9	<10	14.3	-
C (-) „ZPr1996”	0.052	0.074	0.155	6.6	<10	negative	negative
C (+) CR Mab	0.135	0.798	0.551	23.4	nt	nt	nt
C (-) CR Mab	0.048	0.063	0.147	6.3	nt	nt	nt
C (+) Ingezim DAS	2.277	1.987	0.62	26.4	nt	nt	nt
C (-) Ingezim DAS	0.048	nt	0.151	6.4	nt	nt	nt
RHDV2: Ref. 1 (ANSES)	0.585	1.598	0,472	20.1	2560	negative	14.3
RHDV2: Ref. 2 (ANSES)	0.483	1.464	0,673	28.6	2560	negative	16.5

C (+) – positive control; C (-) – negative control; * - percentage OD sample/ OD positive control of ELISA kit; nt – not tested

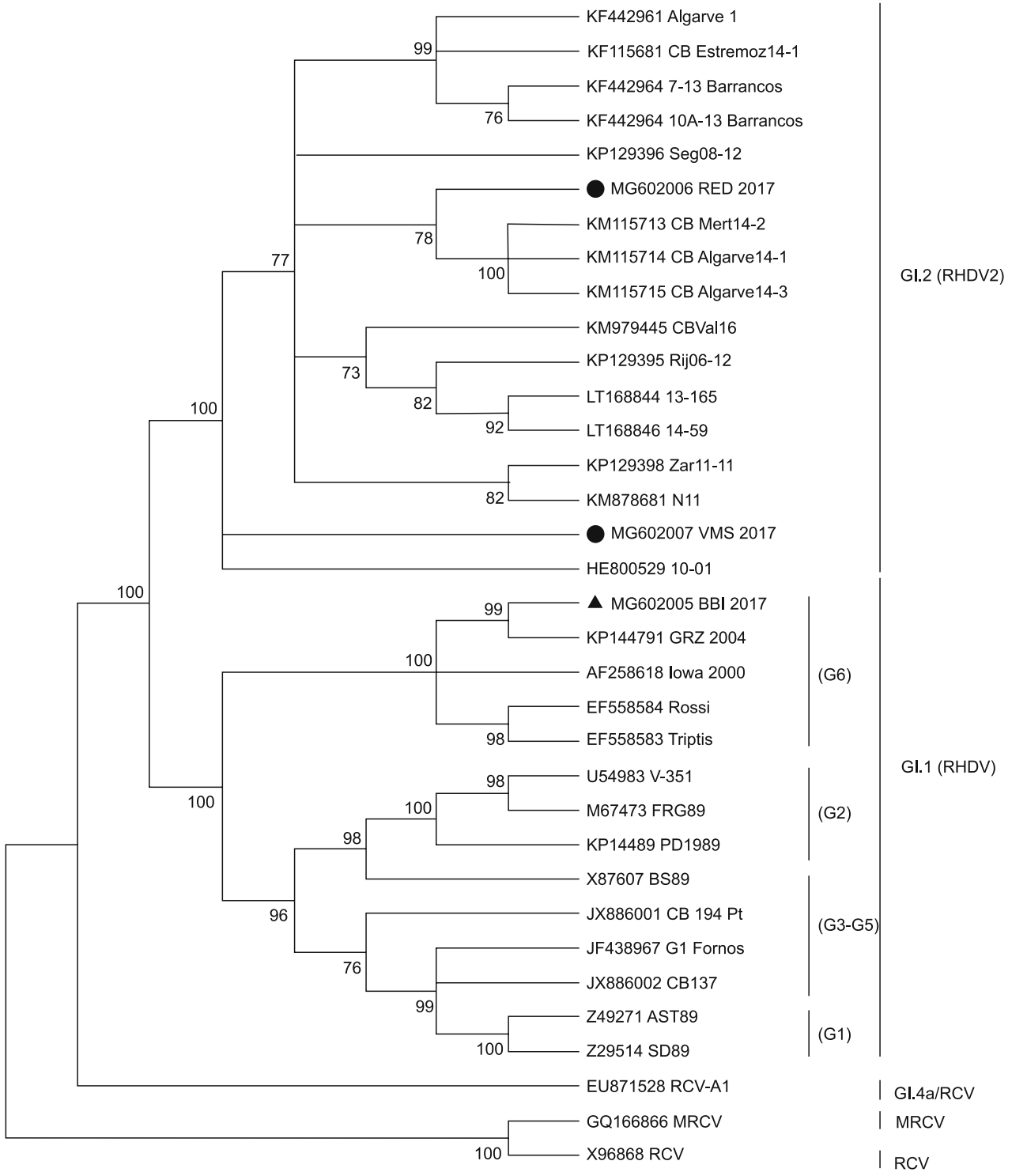
extension step. Cycle threshold (C_T) value (the point on the x-axis showing the number of cycles of replication where the fluorescence breached a threshold fluorescence line) was assigned to all PCR reactions after the amplification. A real-time RT-PCR for the detection of classic RHDV and RHDVa variant was performed as described previously (Fitzner 2014).

RT-PCR, sequencing and phylogenetic analysis

Reverse transcription and cDNA amplification was performed using OneStep RT kit (Qiagen) and panel of specific primer pairs designed according to the published oligonucleotide sequences (Forrester et al. 2006, Le Gall-Reculé et al. 2013, Dalton et al. 2015). The following thermal profiles were applied for RT-PCR: 50°C for 3 min, 94°C for 15 min, 35 cycles of 94°C for 30 s, 57°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min. The same primers were used for sequencing of the amplicons of size from 397 to 897 bp. The PCR products were visualised in 1.5% agarose gel, purified and directly sequenced in both orientations using the ABI Prism BigDye Terminator v3.1 Cycle sequencing kit on an ABI37730x1 DNA sequencer (Life Technologies) at Genomed Sequencing Service as described previously (Fitzner et al. 2012). Based upon overlapping partial nucleotide sequences a 7428 bp complete genome (nucleotide positions 10-7437 according to M67473) each of the three analyzed strains was assembled manually, transformed by Sequin (NCBI) and submitted to GenBank (accesion numbers: MG6002005, MG6002006, MG6002007). For comparative analysis and evaluation of homology of the isolates, BLASTn (Altschul et al. 1990) software was used. The nucleotide sequences of analyzed isolates were compared with RHDV/RHDVa, RHDV2 and RCV sequences retrieved from GenBank (accession numbers pointed in Fig. 1 and Fig. 2). The phylogenetic trees of the *vp60* gene (nucleotide residues 5305-7044) and non-structural genes (NS, nucleotide residues 10-5304) were constructed with MEGA 6 software using Neighbor-joining phylogenetic algorithm (Tamura et al. 2013).

Results

RHDV antigen was detected in all tested samples in used ELISA and HA assays (Table 1). RHDV strains RED 2016, VMS 2017 and BBI 2017 demonstrated heamagglutinating activity with HA titres 10240, 20480 and 10240 respectively. The strongest antigen reactivity of the samples RED 2016 and VMS 2017 was detected in CR Mab ELISA, with OD_{490} 1.5-2.0, i.e. about twice above the positive control. In Ingezim DAS ELISA antigen reactivity of these samples was clearly positive, with OD_{450} of the analyzed samples about twice as high as the cut-off value. In Pab ELISA test, containing

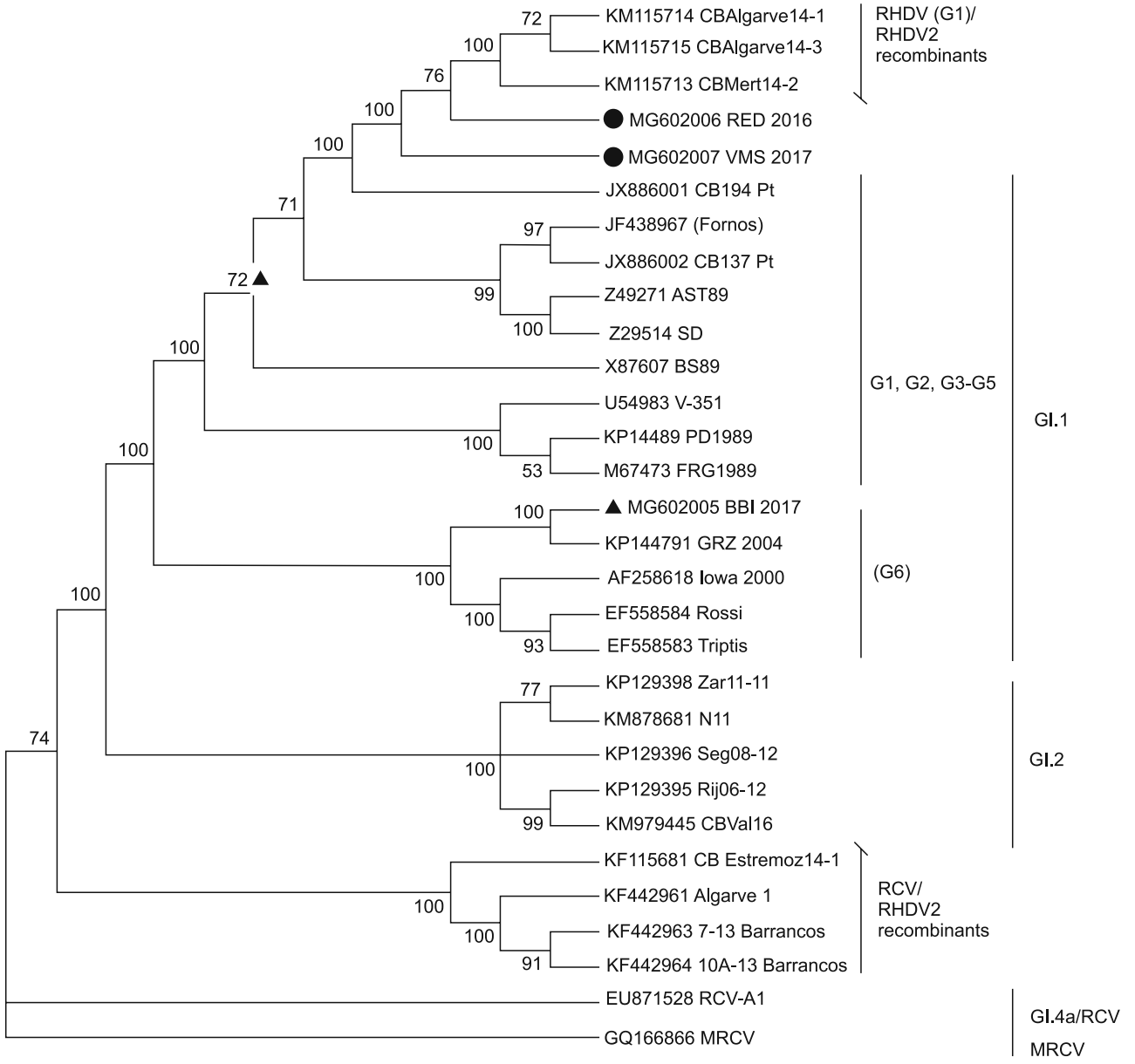


●/▲ indicate Polish RHDV2/RHDVa strains collected in 2016-2017

Fig. 1 Neighbor-Joining tree of 34 RHDV strains based on sequence of VP60 gene. The percentage of replicate trees (>70) in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

hyperimmune sera against classic RHDV of genogroup 2, the result of RHDV antigen detection (OD_{490}) was clearly lower – about two - three times higher of negative control and was similar to the reactivity of two French RHDV2 reference strains. The BBI 2017 antigen response was positive in all ELISA tests and HA. In Pab ELISA the OD reactivity was at the level of positive RHDVa control.

RNA RHDV was detected in all tested field samples. C_T values of all recently delivered RHDV samples



●/▲ indicate Polish RHDV2/RHDVa strains collected in 2016-2017

Fig. 2 Neighbor-Joining tree of 30 RHDV strains based on sequence of non-structural part of RHDV genome (nt 10-5304). The percentage of replicate trees (>70) in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

and RHDV controls in rRT-PCR assay are presented in Table 1. The results of amplification of viral RNA by rRT-PCR assay using RHDV2 probe and specific RHDV2 primers have shown the presence of RHDV variant 2 in liver samples from Reduchów (RED 2016) and Szczecin (VMS 2017), with C_T values in a range 13.5 – 14.5. Similar results were obtained for two reference French RHDV2 strains (C_T values 14.3 and 16.5). RHDV-suspected samples from Białobrzegi (BBI 2017) and all archive classic RHDV, RHDVa and HA-negative RHDV isolates were negative (C_T > 40). However, CT results with classic RHDV-specific probe/primers set (designed according to Gall et al. 2007) showed a high reactivity of BBI strain (C_T values 21-22) and a lack of reactivity (C_T > 35) of the RED 2016 and VMS 2017 RHD virus field isolates and RHDV2 reference strains.

The rRT-PCR results were confirmed by conventional RT-PCR and direct sequencing of the amplified products. Phylogenetic analysis of the complete *vp60* gene showed that strains RED 2016 and VMS 2017 belong to RHDV type 2 (Fig. 1). The RED 2016 and VMS 2017 isolates clustered together with the reference RHDV2 sequences, differently from other Polish

RHDV strains (PD 1989 from RHDV-G2 and GRZ 2004 RHDVa-G6), meaning they represent new evolutionary RHDV linkeages. According to the nomenclature proposed by Le Pendu et al. (2017) these isolates could be termed *Lagovirus europaeus* /GI.2/O.cun/PL/2016/RED and *Lagovirus europaeus*/GI.2/O.cun/PL/2017/VMS. BBI 2017 strain, in contrast to the RED 2016 and VMS 2017, clustered together with RHDVa sequences (G6). However, in the phylogenetic tree of non-structural part of RHDV genome, both Polish RHDV2 strains (RED 2016 and VMS 2017) clustered with RHDV G1 (Fig. 2) near to the Portuguese isolates recently identified as the recombinants of pathogenic RHDV-G1/RHDV2 (Almeida et al. 2015, Lopes et al. 2015).

Intra nucleotide similarity between RED 2016 and VMS 2017 was 97% in VP60 capsid protein and NS proteins. Both Polish RHDV2 isolates showed 82% VP60 nucleotide sequence identity with the reference classic RHDV (FRG89 – M67473), 82% with RHDVa (Rossi – EF558584), 98% with Algarve14-1 (KM115714) recombinant RHDVb/G1RHDV and Algarve1 (KF442961) recombinant RHDVb/European non-pathogenic RCV and RHDV2 strains N11 (KM878681), CBVal16 (KM979445). However, the comparison of RED 2016 and VMS 2017 NS proteins (nt 10-5304) with other RHDV2 sequences revealed 85% similarity, while the homology with classic RHDV/RHDVa reached 90% in this part of genome and was higher than that of RHDVa BBI 2017 (88%).

The first Polish RHDV2 isolates revealed 82% VP60 sequence identity with Polish classic RHDV: (KGM 1988 (KP144790), PD 1989 (KI144789), MAL 1994 (KU882093), BLA 1994 HA-negative (KP144792), OPO 2004 HA-negative (KU882094). The homology of these two native RHDV2 strains with BBI 2017 (MG602005) and other Polish RHDVa-G6 isolates: GRZ 2004 (KP144791), STR 2012 (KF677011), SKO 2013 (KY319034), GLE 2013 (KY319032), BIE 2015 (KY319031) showed about 85% nucleotide sequence identity (82% and 87% in VP60 and NS proteins, respectively). It should be noted that strain BBI 2017 was characterized by 99% (VP60) and 98% (VP10) sequence similarity with RHDVa GRZ 2004 and showed the presence of a rare mutation at position 7026 of the genome that causes that ORF2 start codon is shifted to position 7037, which resulted in the production of a shorter structural protein VP10. To date, this type of mutation has only been demonstrated in the sequence of the Michigan rabbit calicivirus (MRCV) characterized by a weak pathogenicity for rabbits, and in pathogenic RHDV CB137 from G1 (Bergin et al. 2009, Abrantes et al. 2012a,) and has not been diagnosed so far in pathogenic strains of RHDVa subtype.

Discussion

RHD outbreaks were reported among domestic rabbits in Poland since 1988 (Górski et al. 1988).

The results of the recently published phylogenetic study of native RHDV strains, collected between 1988 and 2015, revealed the presence of classic RHDV (G2, G4, G5) and subtype RHDVa (G6) at this time (Fitzner and Niedbalski 2017). The presented studies prove the persistence of RHDV infections in domestic rabbit breeding in Poland. Among the recently disclosed disease cases, RHD outbreak caused by RHDVa was identified, and for the first time the presence of RHDV type 2 was confirmed in samples from two separated cases. Newly detected native RHDV2 strains revealed a similar level of nucleotide sequence differences in *vp60* region, in comparison to the oldest Polish RHDV G2 strains, likewise the Iberian RHDVb in relation to RHDV G1. According to the studies of Duarte al. (2015b) the maximum diversity at nucleotide and amino acid levels observed among RHDV2 VP60 sequences was 3.91% and 2.94%, respectively and can increase in the future. However, phylogenetic analysis of Iberian RHDVb strains from 2012-2014 disclosed the presence of multiple recombination events in the more recent RHDVb genomes, with a single major breakpoint located in the 5' region of VP60. These observations have shown that in contrast to the evolutionary history of older RHDV isolates, recombination plays a key role in generating diversity in recently emerging RHDVb (Lopes et al. 2015). Recombination as a source of variability in RHD virus has already been mentioned in the case of the RHDVa subtype (Forrester et al. 2006) and recombination breakpoints have been described within the capsid and non-structural proteins and between the non-structural and the structural parts of the virus (Abrantes et al. 2008, Lopes et al. 2015, Mahar et al. 2016). The occurrence of recombinant strains RHDV-G1/RHDV2 outside the Iberian Peninsula has been proven both in rabbits and hares in south-western France (Le Gall-Reculé et al. 2017). While the impact of recombination on the fitness of RHDV2 (RHDVb) remains to be assessed, this process has clearly created substantial changes in the viral genome, although whether this led to differences in virulence is unclear.

Since 2010, RHDV2 (RHDVb) has rapidly spread throughout European countries, Africa (Tunisia) (Le Gall-Reculé et al. 2017, World Organization for Animal Health (OIE) Technical disease cards: Rabbit haemorrhagic disease, updated July 2015) and Australia (Hall et al. 2015). The origin of RHDV2 is unclear, however it has been suggested that this virus emerged from a different species, yet unidentified (Le Gall-

-Reculé et al. 2013) and is still adapting to its recent host, the European rabbit and to cross host-species barrier with potential to infect European brown hares and other species of *Leporidae* (Puggioni et al. 2013, Velarde et al. 2016, Le Gall-Reculé et al. 2017, Le Pendu et al. 2017). The latest observations indicate that host range of RHDV2 is broader causing disease not only in rabbits but also in Cape hares (*L.capensis subsp. Mediterraneus*) (Puggioni et al. 2013) and Italian hares (*L. corsicanus*) (Camarda et al. 2014). Recent evidences of RHDV2 infection in brown hares (*Lepus europaeus*) and the development of EBHS-like disease reported in Italy, Spain and France (Velarde et al. 2016, Le Gall--Reculé et al. 2017) support a strong epidemiological potential of this lagovirus. Moreover, in contrast to the previous reports (Dalton et al. 2014, Delibes-Mateos et al. 2014) the latest observations from Italy indicate a high mortality rate of breeding rabbits due to the RHDV2 (Capucci et al. 2017).

To conclude, we detected the first cases of RHDV2 in domestic rabbits in Poland. Amplification of viral RNA, sequencing and subsequent phylogenetic analysis of the *vp60* and NS protein genes, coupled with immunological typing of viral material from samples taken from dead rabbits confirmed the presence of RHDV2 variant. It is also worth noting that these are also the first confirmed cases of RHDV2 in Central Europe. Since the new RHD viruses are located in both phylogenetic trees close to RHDV G1/RHDV2 recombinants, and never before the classic RHDV strains of genogroup G1 were isolated in Poland, it can be assumed that these strains appeared in Poland following the first introduction of RHDV2 of the recombinant type. Route of RHDV2 introduction into Poland is unclear, but given the wide occurrence of RHDV2 globally from the first detection in France, the prolonged survival of this pathogen in organic matter and on fomites, and that RHDV is readily transmitted indirectly, human-mediated spread (Almeida et al. 2015) seems highly possible.

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