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Evaluation of the usefulness of laboratory diagnostic methods in RHD outbreak

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Abstract

The field outbreak of RHD that occurred late summer 2012 on a small-scale rabbit-rearing operation in Poland and the usefulness of techniques for RHD virus diagnosis are described. During the epizootic, the overall mortality rate of rabbits older than two months was 77%. Eight liver specimens collected from dead unvaccinated rabbits (aged 3-5 months) underwent virological examinations. RHDV specific antigen was detected in two out of eight liver homogenates by haemagglutination (HA) test and ELISA, one of the two being negative in HA assay. However the presence of genetic material of RHD virus was confirmed by RT-PCR and real-time RT-PCR in all liver samples tested. Based on antigen reactivity in ELISA and sequencing of PCR amplicons of the *VP60* gene, the RHDVa subtype strain was identified as the cause of infection. The partial genome sequence of a field isolate (STR 2012), comprising the C-terminus of the polymerase gene and the full capsid protein gene, revealed 91% nucleotide homology to reference FRG89 RHDV isolate and 97% to strain Triptis representing the RHDVa variant. Serological evidence of an RHD outbreak in the STR rabbit-rearing operation was confirmed in a serum sample collected from an unvaccinated surviving rabbit. A cross-reactivity examination of RHDV positive serum revealed a decrease in HI titre against the STR 2012 field antigen, and a decrease in the RHDVa control antigen as compared to classic RHDV.

Key words: RHD, diagnostic methods, RHDVa, nucleotide sequence, HI reactivity.

Introduction

Rabbit haemorrhagic disease (RHD) is a contagious and fatal disease affecting domestic and wild rabbits (*Oryctolagus cuniculus*), characterised by an extremely high mortality rate (70-90%) in animals over 6-8 weeks old. The disease, first recognised in China in 1984 (30), has been endemic in many countries of Europe, Asia and in other parts of the world since the end of the eighties (30, 33). Sporadic RHD cases have been described in North Africa (30), in Mexico (19), Cuba, the USA (10) and most recently in Canada (9). In Australia and New Zealand RHD was deliberately introduced in the mid-nineties in order to reduce the population of rabbits (8, 14, 15, 16, 36). Rabbit haemorrgahic disease virus (RHDV), a prototype of the genus *Lagovirus* of the *Caliciviridae* family, is the infectious agent responsible for RHD. The non-enveloped icosahedral virion of RHDV contains single-stranded positive-sense RNA of 7.5 kb. The genome encodes in ORF1 for polyprotein which is then processed to the capsid structural protein (VP60) and nonstructural proteins including helicase, protease, and RNA-dependent RNA-polymerase (RdRp) (28, 32, 38). The gravity of the threat posed by this deadly viral disease has been significantly reduced as a result of studies of the immunogenic and physicochemical properties of RHDV and the development of effective, inactivated RHD vaccines as well as the use of control and preventive measures. However, new outbreaks and new viral strains continue to attract the attention of researchers because of RHDV changeability at the antigenic and genetic level, which may cause some diagnostic problems.

Previous studies have shown the presence of the classic form of RHDV strains with an altered haemagglutination (HA) profile (phenotypic HA-negative variant), RHDV strains known as antigenic and genetic variants (RHDVa), a moderately pathogenic calicivirus (MRCV), a recently new French RHDV variant, and non-pathogenic caliciviruses (RCV, RCV-A1, Lambay, 06-11) (1, 4, 5, 6, 24, 26, 27,

34, 36). Pathogenic RHDV and RHDVa isolates belong to one serotype (2, 3, 10, 36). As the biological and physicochemical properties of the virus have become better known, new diagnostic methods for RHD have been developed. Apart from simple methods for detection of the virus and antibodies specific to the virus antigen, such as the HA and the haemagglutination inhibition (HI) assays, different ELISAs have been developed using polyclonal or monoclonal antibodies (3, 5). Molecular methods such as conventional RT-PCR and real-time PCR have also been introduced for the detection of viral RNA (4, 18, 21).

The first two cases of RHD in Poland were officially diagnosed in the spring of 1988 (20). The clinical symptoms of infection and pathological findings were similar to the description of RHD outbreaks reported at that time in neighbouring countries, and previously in China (29, 33, 35). The isolated RHDV strains - SGM and KGM demonstrate high homology to the German FRG89 and Czech V351 strains (11, 12). In 1994, a non-haemagglutinating phenotypic variant of RHDV was detected (24). New field strains with novel antigenic and genetic characteristics corresponding to Italian and German RHDVa subtype strains were demonstrated in the middle of the first decade of this century (5, 7, 12, 13, 34).

The rabbit breeding system in Poland is still dominated by small-scale rabbit-rearing operations, where animals are bred mainly as an additional source of valuable meat. Currently, the total number of rabbits farmed this way is estimated to be 16 million animals per year. Due to the semi-open production system, the use of portable containers for fresh green feed and water as well as generally poor level of hygiene, the risk of introduction of the RHDV and other pathogens into the population of rabbits' bred is significant.

The aim of this study was to confirm the cause of the fatal rabbit disease, which affected a rabbit farming cottage industry in the southern region of Poland and to display the utility and limitations of the methods used for diagnosis of RHDV infection.

Material and Methods

Animals. The disease occurred on a mixed-breed rabbit farm (STR) in late summer 2012. The stock consisted of fewer than 50 rabbits of various age: five females and one male aged 7-12 months, 20 young rabbits of 3-5 months of age and about 20 unweaned rabbits. According to information gathered during the epizootic, the breeding of rabbits at the premises started over half a year before the outbreak. This had been the first introduction of these animals onto the farm during the past ten years. The parent animals had been purchased on the local market and soon after they had been vaccinated with a monovalent inactivated vaccine against RHD. The rabbits aged 3-5 months and the youngest suckling litters came from offspring of these parents and had not been previously vaccinated. At the same time there were also reports of a similar rabbit disease, with a large number of deaths, in other farms located in close proximity. However, the test samples were not supplied for the laboratory diagnosis.

Specimens. The biological samples were collected from dead rabbits which had contacted RHD without human intervention. They were suspected of having had the acute form of the disease. Eight livers (L1-L8) of infected rabbits from the field outbreak were frozen and provided to the laboratory. The livers were collected from young rabbits (aged 3-5 months) between the first and fourth days of the disease. The liver L8 was collected from last dead 9-week-old rabbit. Prior to testing all livers were examined macroscopically. The serum sample from a surviving rabbit which was 5 months old was collected about three weeks after the last rabbit died.

Virological examinations. To detect RHDV antigen, 20% w/v liver homogenates of diseased rabbits were examined by HA assay with human "0" red blood cells and two ELISAs. One was a sandwich ELISA (PAb) containing of chicken and guinea pig polyclonal antisera against classic RHDV strain, the other was a MAb ELISA, containing of specific antibodies absorbed onto the solid phase and cross reactive monoclonal antibodies (CR) or MAb 1H8 conjugates as tracers (OIE reference laboratory, Italy; code PRRD010130). In both ELISAs the samples were tested at dilutions 1:5 and 1:30, and the OD_{490} nm were compared with positive and negative control antigens.

For genetic analysis conventional RT-PCR and the real time RT-PCR assays have been used. Positive classic RHDV (a 3rd passage of the KGM strain isolated in 1988) and RHDVa variant (GRZ 2004), which were both isolated in Poland, and liver homogenate of healthy rabbits (3-month-old New Zealand white rabbits) as negative RHDV control were included in the studies. The RNeasy Mini Kit (Qiagen) was used for extraction of RNA, which was reverse transcribed for cDNA using oligo(dt)15 primer and AMV reverse transcriptase (Promega).

RT-PCR and sequencing. Specific primers (Table 1) enabling amplification of the 3' end of the RHDV polymerase gene (POL) and the full capsid protein (*VP60*) gene were synthesised according to the primer sequences (4, 5, 15, 21, 22, 25). The following thermal profiles were applied: 94°C for 3 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. Amplicons were visualised by 1.5% agarose gel electrophoresis, 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, formerly Applied Biosystems, USA) in Genomed Sequencing Service and Synthesis IBB (Polish Academy of Science).

Overlapping sequences of amplicons were used to establish the consensus nucleotide sequence (2514 nucleotides long) of STR 2012 RHDV field strain. For comparative analysis and evaluation of homology of the isolates, BLASTn and MEGA5 software were used. The phylogenetic tree based on the 786 nucleotide fragment of RHDV *VP60* gene was constructed using the neighbour-joining method with bootstrap value 1000 (37, 39). In total, 26 sequences available in GenBank (including 10 RHDV and 14 RHDVa isolates and two non-pathogenic caliciviruses), 11 previously sequenced Polish RHDV strains (13), and the STR 2012 sequence were analysed.

Real-time reverse transcription (rRT-PCR). P7 forward primer (6941-6961) ACYTGACTGAACTY ATTGACG, P8 reverse primer (7044-7022) TCAGACATAAGAAAAGCCATTGG, and the TaqMan probe (6986-7010) FAM-CCAARAGCA CRCTCGTGTTCA ACCT-TAMRA according to Gall *et al*. (18) were used. The assay was performed in the 7300 Fast System Real Time thermal cycler (Life Technologies, formerly Applied Biosystems, USA) as a one-step reaction using the QuantiTect Probe PCR Kit (Qiagen, USA). The reaction mixture at the volume of 25 µL contained: 5 µL of extracted RNA; 12.5 µL of 2 x QuantiTect Probe RT-PCR Master Mix; 0.25 µL of QuantiTect RT Mix; 0.5 µL (2.5 pmol) of Taqman probe; 1.0 µL of forward primer; 1.0 µL of reverse primer, 1.25 µL of $MgSO_4$, and 3.5 µL of H_2O. Both primers were at a concentration of 10 pmol. The cycling conditions were as follows: I – reverse transcription at 50°C for 30 min; II – reverse transcription inactivation and Taq DNA polymerase activation at 94°C for 2 min ; III – 45 cycles of PCR at 94°C for 30 s, 55°C for 45 s, and 68°C for 45 s (8). The reporter dye (FAM) was measured at the annealing stage in the exponential phase of the amplification plot of each cycle. After completion of the PCR, threshold-crossing values (Ct) were established automatically at 0.2 level. A cycle threshold value below 35 was considered positive, as compared to RNA of KGM RHDV strain. The SVDV RNA, RNA isolated from the liver of a healthy rabbit, and water DEPC were included as negative controls.

Serological analysis. For detection of RHDV specific antibodies and cross reaction, convalescent serum and the control sera were tested by HI assay. For confirmation of the results of HI, ELISAs were performed using the liquid-phase blocking ELISA (LPBE) kit containing polyclonal RHDV specific antibodies and the inhibition MAb ELISA kit (IZSLER, Italy, code PRRD010110).

Reference positive control. Nine polyclonal immune rabbit sera from the laboratory collection were used. Four anti-RHDVa sera (20/0309, 21/0309, 24/0309, 25/0309) were obtained 14 d after vaccination (pv) of seronegative (HI/ELISA titre <20) New Zealand white rabbits with experimental inactivated vaccines containing GRZ and KRY RHDVa antigens. Four anti-RHDVa sera were obtained from the same rabbits after a challenge with the homologous virus strain (12). Highly positive RHDV rabbit serum (1/1095) was produced after two consecutive immunisations with purified VP60 protein of KGM strain.

HI. The test was carried out on a U-bottomed microtitration plate. Before testing, the sera were inactivated at 56°C for 30 min and treated with a 25% solution of caolin at 25°C for 20 min. Serial two-fold dilutions of serum samples, starting from 1:10 were performed in a volume of 50 µL in 0.85% phosphate buffered saline (PBS) (pH 7.5), mixed with an equal volume of viral antigen for 30 min at 18-26°C, and incubated with 100 µL of 0.75% human red blood cells suspension (type "0") at 18-26°C for 1.5 h. The antibody level was measured against 4 HA units of classic RHDV (KGM), subtype RHDVa (KRY and GRZ), and the haemagglutinating extract L7 of STR field isolate, and expressed as the reciprocal of serum dilution with full inhibition of haemagglutination.

Table 1. Description of oligonucleotide primers used for amplification of the C-terminal part of the RHDV polymerase gene and the complete *VP60* gene

Primer set	5' position* (nt)	Sequence	Product size (bp)	Genome region
S1 (F)	4510-4530	5' GACTACTCAAAGTGGGACTCC	860	POL / VP60
S10 (R)	5393-5378	5' ATGCCATCGGTTGTGG		
P1 (F)	5182-5201	5' GAGCTCGAGCGACAACAGGC	511	POL / VP60
P2 (R)	5692-5671	5' CAAACACCTGACCCGGCAAC		
N3 (R)	5226-5206	5' ACCCAGTTCAGTGTTTACAGC	465	POL / VP60
N4 (F)	5671-5650	5' CTATGAAGCGAAACTGCATGCC		
P3 (F)	5311-5334	5' GGCAAAGCCCGTACAGCGCCGCAA	519	VP60
P6 (R)	5829-5811	5' GGGACGCAAGTCTGGCATG		
P11 (F)	5682-5698	5' AGGTGTGTTTGGTGGGC	434	VP60
P4 (-)	6116-6097	5' AACCCTCCAGGTACTGGTTG		
SP5 (F)	6069-6088	5' CAGGTGGAACGGCCAAATAG	585	VP60
SP7 (R)	6654-6634	5'AGGAGTGCCGGGTGTGGTTAC		
SP15 (F)	6301-6321	5' CCAGACGGCTTTCCTGACATG	593	VP60
SP13 (R)	6894-6874	5' CACACTTAAACCAATCTCCAT		
P12 (F)	6607-6626	5' ACATACACACCTCAACCAGA	464	VP60
P5 (R)	7071-7051	5' GCACCTGCAAGTCCCAATCCG		

* - according to FRG89 RHDV strain (accession number M67437)

Liquid-phase blocking ELISA (LPBE). LPBE, a laboratory kit containing polyclonal RHDV specific antibodies, was used. Briefly, the ELISA plates (Maxisorp, Thermo Scientific, USA) were coated with a hyperimmune hen RHDV antiserum in carbonate/ bicarbonate buffer (pH 9.6) for 18-24 h at 2-8°C and used for the test (or stored frozen at -20°C). On the first day, a dilution series of tested /control serum samples were incubated on a U-bottomed microtitration plate (liquid phase) with an equal volume of constant pretitrated RHDV antigen (strain KGM) in PBS-Tween buffer (pH 7.4) for 18-24 h at 2-8°C. On the second day, 50 µL of the mixture was transferred onto the coated microplate ELISA and then incubated at 37°C for 60 min. In the next stage, antibodies (guinea pig- RHDV antiserum) were added and incubated for 60 min at 37°C. RHDV-specific antibodies were detected, using horseradish peroxidase (HRP) conjugate anti-guinea pig IgG, after incubation for 60 min at 37°C. Finally, the o-phenylenediamine (OPD) solution in a phosphate/ citrate buffer, pH 5.0 was added and then the reaction was stopped. A second ELISA was performed using the commercial inhibition MAb ELISA kit (IZSLER, Italy, code PRRD010110) according to the manufacturer's instructions.

In both ELISAs, the sera were diluted from 1:10 to 1:20 480 (final concentrations). The titre of antibodies against RHDV was expressed as the reciprocal $\log_{10}$ of final dilution of serum with 50% of the OD_{490} nm of control antigen.

Results

The course of the disease was very dramatic with high morbidity and near 77% mortality rate in animals older than two months (90% of young unvaccinated animals). The clinical signs seen in the agonal phase of diseased rabbits, such as squeaking, convulsions, respiratory difficulties, dullness, and in a few cases bloody nasal discharge, suggested rabbit haemorrhagic disease. Within a week, 20 rabbits died: the parent buck and doe and 18 non-vaccinated young rabbits. Four does, two non-vaccinated rabbits from the age group of 3-5 months and all the suckling rabbits survived the epizootic. Macroscopic examination of livers of rabbits which died after natural infection with STR RHDV revealed necrotic hepatitis. The livers 1-7 weighed from 80 to 90 g, while liver 8 weighed 32 g. The livers 1-6 and 8 were brown and fragile with soft tissue consistency and with congested gallbladders.

Single white nodules of diameter 1-2 mm were observed on the surfaces of livers 3 and 4, pointing to coccidiosis. Liver 7 was of a soft consistency with rounded edges, beige, but in some places yellowish pointing to jaundice. The results of comparative diagnosis with the virological methods are presented in Table 2. Only the L7 extract always demonstrated heamagglutinating activity of 0.75% human "0" red blood cells irrespective of the conditions used (*e.g.*: buffer pH 6.5 or 7.5, time and temperature of incubation: 2 h at RT or over night at 2-8°C).

Table 2. Results of RHDV detection by RT-PCR, rRT-PCR, HA, and ELISA in rabbit livers (L)

Methods		Liver							
		L1	L2	L3	L4	L5	L6	L7	L8
RT-PCR	amplicon								
	S1 – S10 860 bp	(-)	(+)	(+)	nt	(+)	(+)	(+++)	(+++)
	P1 – P2 510 bp	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
	N3 – N4 465 bp	(-)	(-)	(-)	(-)	(-)	(-)	(++)	(++)
	P3 – P6 519 bp	(+)	(+++)	(+++)	(++++)	(++++)	(++++)	(++++)	(++++)
	P11 – P4 434 pz	(+++)	(+++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)
	SP5 – SP7 585 bp	(++)	(++)	(+++) (++++)	(+)	(++)	(+++)	(++++) (++++)	(++++)
	SP15-SP13 594 bp	(+)	(++)	(++++) (++++)	(++++)	(+++)	(++++)	(++++) (++++)	(++++)
	P12 – P5 464 bp	(++)	(+++)	(++++)	(++)	(++++)	(+++)	(++++)	(++++)
rRT-PCR	Ct	30.42	31.06	26.93	32.2	28.63	26.86	17.14	17.32
HA	pH 6.5	(-)	(-)	(-)	(-)	(-)	(-)	10240	(-)
	pH7.5	(-)	(-)	(-)	(-)	(-)	(-)	20480	(-)
ELISA	PAb	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)
ELISA	MAb CR	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)
	1H8	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)

(-) – negative,(+) – positive, (+ /++++) – intensity of the band, nt – not tested

Estimated HA titres varied from 10 240 (0.85% NaCl, pH 7.5) to 20 480 (PBS, pH 6.5). Liver extracts 1-6 and 8 did not demonstrate agglutination activity. Using the laboratory sandwich ELISA, the RHDV antigen was detected in two out of eight liver homogenates tested. The recorded OD_{490} nm at 1:5 dilution reached about 40%-60% value of the positive control KGM antigen for L7 and 75%-85% for L8. RHDV antigen was not detected in livers 1-6. Similar results were obtained using MAb ELISA kit with CR conjugate, with the exception that the specific antigen reaction of L7 ranged from 60% to 90% of the positive control and the reaction of L8 ranged from 25% to 55%. When using MAb 1H8 conjugate raised against classic RHDV instead of CR conjugate, no specific RHDV antigen was detected in any of the tested livers. All field samples submitted for examination were recognised as RHDV positive by real-time RT-PCR. The highest concentration of viral RNA was demonstrated in livers 7 and 8. Ct values of non diluted RNA for samples L7 and L8 were 17.14 and 17.32 respectively. In the samples L1-L6, Ct values were significantly lower, ranging from 27 to 32. The Ct of positive control (KGM) RHDV was detected at 16.5 value. The Ct values of negative control - SVDV RNA, RNA from a healthy rabbit, and the water sample - were higher than 40.

Using conventional RT-PCR method, specific products of RHDV genome were obtained with cDNAs of samples L7 and L8, except for P1-P2 primer set. For cDNA, the specific amplicons were visualised in samples from rabbits 1-6 in 1.5% agarose gel only with primer sets dedicated to *VP60* gene. When the reaction was performed with primers specific for the RNA polymerase gene (S1-S10) or a portion of *VP60* gene (N3-N4), the bands were less intensive or invisible. No band was observed for the L1-L8 cDNA with P1-P2 primer set (Table 2) whereas, specific bands of expected size of 510 bp were detected for RHD positive control - KGM strain.

Sequence analysis of six genome fragments of RHDV field isolate revealed 100% sequence identity of RNA samples derived from dead rabbits 7 and 8. Based on overlapping sequences of the L7 and L8 amplicons.

A consensus 2514 nucleotide sequence (positions 4531-7044) of RHDV field strain STR 2012 was established (Fig. 1) (GenBank accession number KF677011). Comparison of the nucleotide sequence of STR 2012 isolate to the reference classic RHDV FRG89 strain (M67473) and the reference RHDVa isolate Triptis (EF558583) to the classic M67473 showed overall similarity of 91% and 92% respectively. The homology decreased to 87% and 88% respectively in the 3' region of the polymerase gene. Overall 8% and 6% variability of the nucleotide sequence of STR and Triptis isolates, as compared to FRG89, was demonstrated in the capsid protein gene. Additionally, STR 2012 and Triptis isolates share 97% of nucleotide sequence homology in the entire analysed genome fragment. The similarity of the same portion of the STR 2012 genome to the Polish classic strain KGM RHDV is 92%, and 90% to the Polish BLA HA-negative isolate. Analysis of deduced amino acid sequences revealed 3.6% total variability of the STR isolate in relation to the FRG89 strain.

RHDV antibodies with high ELISA and HI titres were detected in the serum of a surviving rabbit, confirming recent infection with RHD virus in the STR infected rabbit-rearing operation (Table 3). STR 2012 convalescent rabbit serum cross-reacted in the HI assay with the homologous antigen as well as with antigens representing classic RHDV and RHDVa subtype strains. The HI titre of specific antibodies against the STR antigen was only slightly higher compared to the KGM antigen. Similar results were also obtained using two RHDVa positive antigens (GRZ and KRY). However, clear differences in detectable RHD antibodies levels were found in the HI assay when reference RHDV and RHDVa positive control sera were titrated against RHDV, RHDVa or STR 2012 antigens. Cross-reactivity examination of rabbit hyperimmune serum 1/1095 obtained after immunisation with highly purified KGM VP60 protein revealed HI antibodies titre of 20,480 to the homologous antigen and a significant decrease (32 to 64 fold) in titre against RHDV STR liver extract or RHDVa antigens. In turn, four RHDVa reference antisera (20/0309, 21/0309, 24/0309, and 25/0309) exhibited similar levels of HI detectable antibodies with homologous antigens and STR 2012 field antigen, and a 4-32 fold decrease in HI titre to KGM virus. These antisera were obtained two weeks after vaccination of rabbits with experimental vaccines containing GRZ or KRY antigens. The HI titres of sera obtained after a challenge with homologous virus strains ranged from 5,120 to 20,480. However, the differences in HI cross-reactivity using both types of RHDV antigens were not so significant and did not exceed the range of two dilutions.

Discussion

The structure of rabbit farming in Poland is dominated by small backyard farms. In this type of breeding there is a seasonal increase in the population of rabbits from spring to autumn. In these farms, due to the high fertility of rabbits and the short growth period as well as the emergence of a new generation of fully susceptible young rabbits, the risk of an outbreak of RHD is particularly large. At the same time, small farm breeders are the main recipients of inactivated vaccine against RHD. Currently, this market's size is estimated at at least 1.5 million doses per year. In counterpoint to this, intensive commercial rabbit breeding for meat, although much more expensive and demanding, is gradually expanding.

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4531    ACTATGTCACCGTGCGTTGTTAGGTTGGCAATTGACATCTTAGCTGACTGTTGTGAACAG  4590
        ACGGAACTTACCAAAAGCGTCGTACTCACATTAAAGTCCCACCCCATGACCATACTTGAC  4650
        GCCATGATCGTACAGACCAAACGTGGTCTCCCGAGCGGCATGCCCTTCACTTCGGTCATC  4710
        AATTCTATCTGCCATTGGTTACTTTGGTCTGCAGCCGTTTACAAGTCTTGTGCTGAGATT  4770
        GGTCTACACTGTTCCAACCTGTATGAAGATGCCCCATTCTACACGTACGGTGACGACGGC  4830
        GTGTATGCCATGACTCCGATGATGGTAAGCCTTTTACCTGCAATAATAGAGAACTTGAGA  4890
        GACTACGGCTTGTCTCCCACTGCCGCAGACAAAACAGAGTTTATAGATGTTTGCCCGCTC  4950
        AACAAGATCTCGTTTTTGAAGCGCACTTTTGAGCTCACGGACATCGGGTGGGTGTCAAAG  5010
        TTAGACAAATCTAGCATACTCAGGCAGCTGGAGTGGAGTAAAACAACATCCAGGCACATG  5070
        GCGATTGAGGAAACATATGACCTAGCAAAGGGAGAACGTGGTGTGCAGCTAGAGGAGCTA  5130
        CAGGTTGCCGCTGCCGCACATGGCCAGGAGTTTTTCAGCTTTGTCCGCAAAGAACTTGAA  5190
        CGGCAACAAGCATACACTCAGTTTAGTGTTTATAGTTATGATGCTGCTAGAAAGATCCTT  5250
        GCAGATCGTAAGAGAGTCGTCTCGGTGGTACCTGACGACGAATTTGTGAATGTTATGGAG  5310
        GGCAAAGCCCGCACAGCGCCGCAAGGCGAAGCAGCAGGCACTGCTATCACAGCATCAGTT  5370
        CCCGGAACCACGACCGACGGCATGGATCCTGGTGTAGTGGCCGCAACTAGTGTGGTCACT  5430
        GCAGAAAATTCATCCGCATCGGTTGCAACGGCGGGGATTGGCGGCCCACCCCAACAGGTG  5490
        GACCAACAAGAGACATGGAGGACAAACTTTTACTATAATGATGTTTTCACTTGGTCCGTC  5550
        GCGGATGCGCCCGGCAGCATTCTCTACACTGTCCAACACTCCCCACAGAACAACCCATTC  5610
        ACAGCCGTACTGAGCCAGATGTACGCTGGCTGGGCTGGTGGCATGCAGTTCCGTTTCATA  5670
        GTTGCTGGATCAGGTGTGTTCGGTGGGCGACTGGTCGCGGCTGTGATACCACCAGGCATT  5730
        GAGATTGGACCAGGGTTGGAGGTCAGGCAATTTCCTCATGTTGTTATCGACGCCCGTTCA  5790
        CTCGAACCTGTTACCATCACCATGCCAGACTTGCGTCCCAACATGTACCATCCAACTGGT  5850
        GACCCTGGCCTTGTCCCCACACTAGTCCTCAGTGTTTACAACAACCTCATCAACCCGTTT  5910
        GGTGGGTCCACCAACGCAATCCAGGTAACGGTGGAAACGAGGCCGAGTGATGATTTTGAG  5970
        TTCGTGATGATTAGAGCCCCCTCCAGCAAAACTGTTGACTCAGTCTCACCCGCAGGCCTC  6030
        CTCACGACCCCAGTCCTCACTGGTGTTGGCAATGACAACAGGTGGAACGGCCAAATAGTG  6090
        GGACTGCAACCAGTACCTGGGGGGTTTTCCACGTGCAACAGGCATTGGAACCTGAACGGC  6150
        AGCACATATGGCTGGTCAAGCCCTCGGTTTGCCGACATTGACCATCGAAGAGGCAGTGCA  6210
        AGTTACTCTGGGAACAACTCCACCAACGTGCTTCAGTTTTGGTACGCTAATGCTGGGTCT  6270
        GCAATTGACAATCCTATCTCCCAGGTTGCACCGGACGGCTTCCCTGACATGTCATTCGTG  6330
        CCCTTTAACAGCCCCAACATTCCGACCGCGGGGTGGGTCGGGTTTGGTGGTATTTGGAAC  6390
        AGTAACAACGGTGCCCCCGCTGCTACAACTGTGCAGGCCTATGAGTTAGGTTTTGCCACT  6450
        GGGGCACCAAATAACCTCCAACCCACTACCAACACTTCAGGTGCACAGACTGTCGCTAAG  6510
        TCCATTTATGCCGTGGTAACTGGCACAAACCAAAATCCAACCGGACTGTTTGTGATGGCC  6570
        TCGGGTGTTATCTCCACCCCAAACGCCAGCGCTGTCACATATACGCCCCAACCAGACAGA  6630
        ATTGTGACTACACCCGGTACTCCTGCCGCCGCACCTGTGGGTAAGAACACACCCATCATG  6690
        TTCGCGTCTGTTGTCAGGCGCACCGGTGACGTCAACGCCACAGCTGGGTCAACCAACGGG  6750
        ACCCAGTACGGCACGGGCTCCCAACCACTGCCAGTAACAATTGGACTTTCGCTCAATAAC  6810
        TACTCGTCAGCACTCATGCCTGGGCAGTTTTTCGTTTGGCAGTTAACCTTTGCATCTGGT  6870
        TTCATGGAGATTGGCTTAAGTGTGGACGGGTATTTTTATGCAGGAACAGGAGCCTCAACC  6930
        ACGCTCATTGACTTGACTGAACTCATTGACGTACGCCCCGTGGGACCCAGGCCGTCCAAG  6990
        AGCACACTTGTGTTCAACCTGGGGGGCACAACCAATGGCTTTTCTTATGTCTGA        7044
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Fig. 1. Nucleotide sequence of the RHDVa field strain STR 2012 covering 3' part of the polymerase gene and entire capsid protein gene (2514 nt)

Table 3. Detection of RHDV antibodies in the sera of convalescent and vaccinated rabbits by HI test and ELISA

Tested sera		Titres					
		HI (antigens)				LPB ELISA	MAb ELISA (1H8)
		STR2012	GRZ RHDVa	KRY RHDVa	KGM RHDV		
STR 2012 - convalescent		5120	2560	2560-5120	1280	5120	10 240
GRZ	20/0309 pv	1280	640	640	40	40	80-160
	21/0309 pv	320	160	80	20	10	40
KRY	24/0309 pv	1280	1280	1280	160-320	160-320	640
	25/0309 pv	640	640	640	160	80	160
KGM	1/1095 hp	640	640	320	20 480	10 240	20 480

pv – 14 days post vaccination hp – hyperimmune serum anti-VP60 KGM RHDV

In addition to maintaining good general conditions of rabbits, appropriate hygiene and disinfection measures, and a controlled feeding system, commercial farms use prophylactic systematic vaccination against RHD and myxomatosis, usually involving the females of stock animals. All these measures better prevent accidental infection by RHDV.

Currently, new cases of RHD in Poland are reported much less frequently, which certainly does not mean that the disease has disappeared or that the RHDV has ceased to circulate. Observations over the last several years demonstrate that periodically RHD occurrence increases. Infections occur especially on the smallest premises with poor zoosanitary conditions, which can be a reservoir of the virus. It is likely that due to the large number of farms maintaining a relatively small number of animals (under 50 rabbits) and due to the additional costs of veterinary care, some of the outbreaks are not reported by breeders.

In the study, the RHD outbreak that affected a total 77% of young and adult rabbits over two months was caused by RHDVa subtype strain that was confirmed by multiple laboratory diagnostic methods. However, despite the obvious clinical signs of the disease, RHD virus could be demonstrated in only two out of eight livers submitted for testing by HA test and ELISA. These conventional diagnostic methods thus only partially confirmed the clinical diagnosis of infection. It can be explained by the earlier vaccination of the does and the possible role of maternal antibodies. Haemagglutinating antigen was identified by ELISA, indirectly indicating RHDVa subtype strain as the presumed causative agent of the outbreak. The diagnosis was confirmed by serology but finally only the results of genetic tests allowed the full identification of the type of RHDV responsible for the epizootic. Although this new Polish strain and previously isolated native RHDVa strains are separated by at least 6 years in their discoveries and all were isolated at various geographically distant sites, they exhibit a very similar phylogenetic profile. Analysis of the genetic homology of the presented field isolates and previously described native RHDVa strains (13) showed that strain STR 2012 belongs to the RHDVa subgroup and exhibits the greatest similarity to strains GRZ and DCE. The level of genetic variability between the STR 2012 isolate and the group of native RHDVa strains, in a fragment encompassing the two variable regions C and E of the capsid protein gene according to Neill (31), ranged from 3 to 4 % except 5% for KRY isolate.

It is worth noting that also in the conventional RT-PCR method some differences in the reactivity of individual samples and ability to detect the genetic material of RHDV have been observed. In addition to the differences in detection and reactivity of the STR 2012 RHDV by conventional laboratory diagnostics (HA, ELISA) our study showed limited utility of some of the primers for cDNA amplification. The study showed the lack of amplification of STR 2012 genetic material with P1 primer (5182-5201) from the C-terminus of the RHDV RdRp polymerase gene. The primer set P1/P2, originally proposed by Guittré *et al.* (21), has been used for many years in many laboratories for routine amplification of classic RHDV strains, including HA-negative phenotypic variants (23, 26). Similar difficulties were encountered in the amplification of cDNAs of the samples L1-L6 with the pair of primers N3-N4 encoding the N-terminal part of the *VP60* gene. For L7-L8 homogenates the problem was overcome by amplifying a larger fragment (860 bp) of the genome comprising the polymerase gene and the *VP60* gene, with the use of primers S1-S10. The analysis of the short fragment of polymerase gene from the positions 5175 to 5229 revealed two and three additional nucleotide mutations as compared to Triptis and GRZ isolates respectively. Furthermore, compared to the strain FRG89, in this fragment of the sequence four nucleotide mutations occur immediately prior to primer P1 and other 10 within or just outside the primer, which may explain the lack of amplification.

According to the alignment nucleotide sequences of a 2514 bp fragment, multiple mutation sites of STR 2012 isolate were found in the 3' end of the polymerase gene as well as in the nucleotide sequences located in hypervariable regions C and E of RHDV *VP60* gene. The large accumulation of nucleotide point mutations in the carboxy-terminus of the polymerase gene resulted in a replacement of 5 amino acids and 25 amino acids in the *VP60* gene (numerous nucleotide substitutions resulting in amino acid substitutions were disclosed in the highly variable regions C and E of the capsid protein gene). Further analysis of genetic homology, covering both highly variable regions, indicated close overall similarity of the STR isolate to the previously described Polish RHDVa isolates.

Thus nucleotide sequence similarity in the fragment from nucleotide position 4820 to 7044 revealed 98% identity of the STR 2012 isolate with GRZ and DCE isolates and 97% with German Triptis RHDVa. Phylogenetic analysis of a 786 nucleotide fragment of the *VP60* gene of the STR 2012 isolate, and the sequences of different representatives of RHDV as well as RHDVa isolates from GenBank, demonstrated the similarity of this new isolate with RHDVa subtype group (Fig. 2). A comparison of the deduced amino acid sequences confirmed the close relationship of this new field isolate with Italian (Pv97, Vt97), German (Hartmansdorf, Triptis) and previously described Polish RHDVa isolates (5, 13, 34).

Based on antigenic and genetic characteristics, pathogenic RHDV strains are currently subdivided into two main groups comprising: classic RHDV and RHDVa subtype strains (5, 34). Numerous reports from the last decade concerning RHDV strains, collected around the world, perfectly reflect the spread of the RHDVa subtype. Examinations of RHDV strains isolated in Poland, over a similar period, clearly confirmed the emergence of the antigenic and genetic RHDVa subtype in our country (7, 12, 13). The results of the study support the primary hypothesis of the displacement of the original RHDV by the strains belonging to the RHDVa subtype. The STR 2012 isolate was found to be closely related to previously described Polish RHDVa strains, indicating endemic maintenance and circulation of RHDVa in the environment. It should be underlined that the RHDVa subtype strain has been isolated for the first time in this region, despite the favourable conditions for its spread which relate to the nature of rabbit breeding.

Genetic mutations/recombinations and changes in the antigenic properties have an impact on further differentiation of the strains of RHDV. Evidence of this is the emergence of the decreasingly homogenous new strains with altered antigenic and genetic features, showing different tropism and pathogenicity for the

target species. The strains detected in relatively short period of time, which demonstrate new characteristics, *vide* phenotypic HA-negative RHDV variant, antigenic and genetic RHDVa variants, and the new American and French RHDV variants, are examples of this phenomenon (5, 6, 8, 17, 24, 27, 34, 36). This may be due to the effect of environment, the use of vaccination, or the process of evolution. It is well known that RNA viruses show a far-reaching capacity to reemerge in new forms much different from the original patterns. The lack of any proof-reading mechanism for RHDV polymerase gene facilitates the occurrence point for mutations during replication. All these factors can lead to the formation of new variants of existing forms of RHD virus, with modified characteristics, and probably more appropriate to the new conditions. This may in turn pose serious diagnostic problems and require the development of new vaccines, containing the current more immunogenic virus strain.

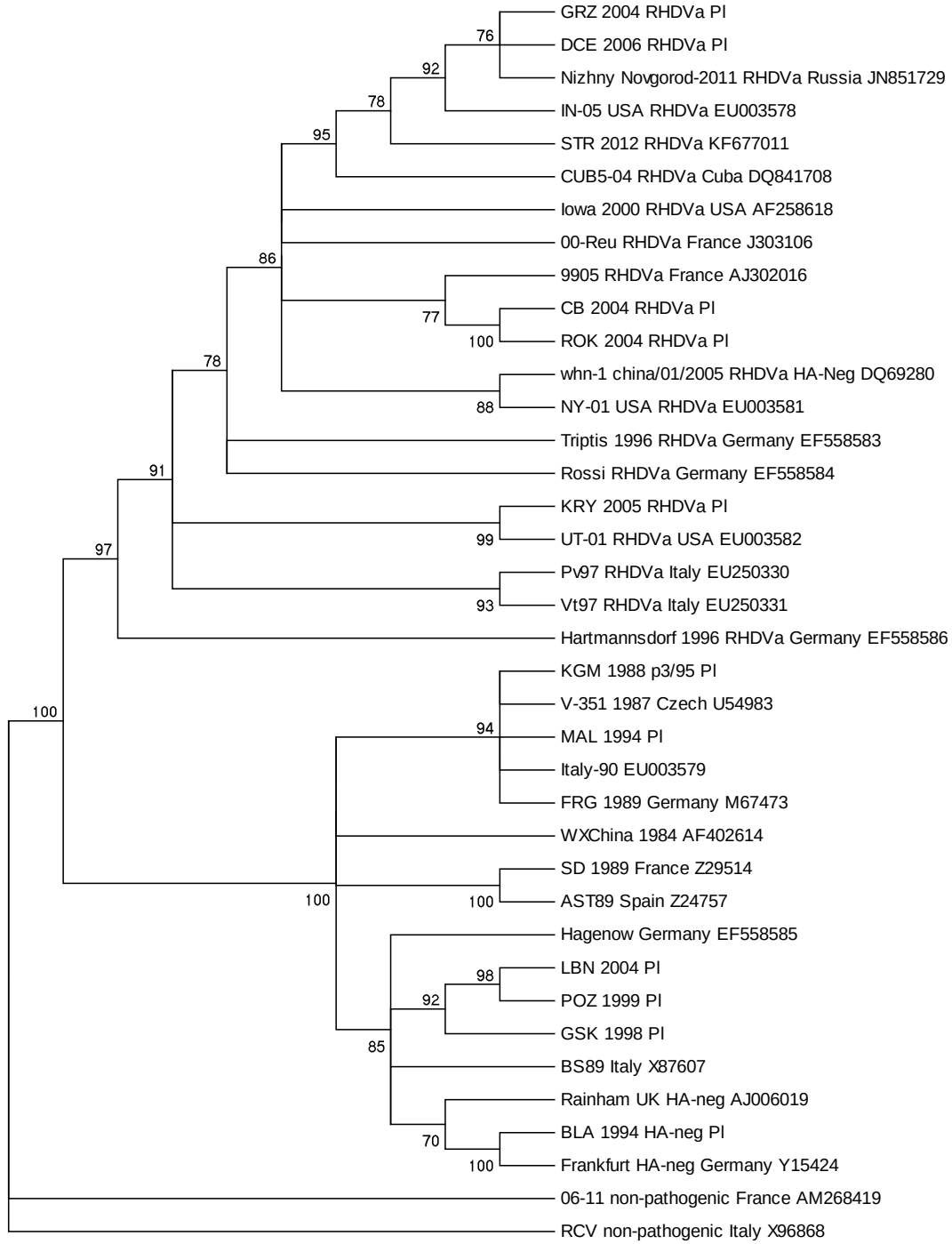

Fig. 2. Phylogenetic tree of 38 RHDV isolates made by MEGA5 software, using neighbour-joining method with bootstrap values calculated for 1000 replicates. The analysis was conducted on fragment composed of 786 nucleotides (6088-6873), encompassing the most variable part of capsid protein gene. The bootstrap values greater than 70% are given before each branch node

Interesting observations have been also provided by serological examination. Testing the convalescent serum by ELISA and HI test showed the presence of specific antibodies of high titres, characteristic for RHD infection. Furthermore, the results of HI revealed differences of antibody titres depending on the type of viral antigen used in the study. Although all tested sera cross-reacted with both groups of antigens (homologous *vs* heterologous), the study demonstrated significant differences of immunological response, reflecting antigenic differentiation of RHDV strains.

On the basis of the study, the importance of applying a number of the tests for diagnosis of RHD should be underlined. It is equally important to regularly check and update the tests used in the diagnosis, including PCR assays. This approach requires constant observation of the genetic evolution of the RHD virus strains circulating in domestic and wild rabbit populations.

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