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DETECTION OF RABBIT HEMORRHAGIC DISEASE VIRUS (RHDV), GENOTYPE GI.1 AND GI.2 WITH A NEW MULTIPLEX REAL-TIME RT-qPCR PROTOCOL, USING THE MINOR CAPSID VP10 GENE

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Aim. This study aimed to develop a specific and sensitive multiplex real-time reverse transcription PCR (RT-qPCR) method for the detection and differentiation of rabbit hemorrhagic disease virus (RHDV) genotypes GI.1 and GI.2 circulating in Ukraine, using primers and probes based on the ORF2 gene, encoding the minor capsid protein VP10. Furthermore, to perform an initial validation of this PCR. **Methods.** The assay was designed to amplify a conserved 101-bp ORF2 sequence, encoding the minor capsid protein VP10, assuming that it would be a less variable region compared to that of the traditionally targeted ORF1 sequence encoding the major capsid protein VP60. Sequence alignment was performed using 38 full-genome sequences of RHDV isolates of various geographic origins present in GenBank. Specific primers to RHDV and two genotype-specific hydrolysis probes (FAM for genotype GI.1 and HEX for genotype GI.2) were designed and partially validated both *in silico* (BLAST) and *in vitro*. RNA was extracted from 6 tissue samples (contaminated with RHDV and negative control), and two virus reference strains using the IndiSpin Pathogen Kit, followed by RT-qPCR using a one-step protocol. This protocol and primer and probe sequences are detailed in the main text. Analytical sensitivity and specificity were assessed by a 10-fold RNA serial dilution (from 10^6 to 10^0 copies). For specificity testing, in addition to RHDV isolates, non-target organisms were included: *Myxoma virus* (strain B-82, IVM NAAS collection), *Staphylococcus aureus* subsp. *aureus* ATCC 25923, *Pasteurella multocida* subsp. *multocida* ATCC 12945, *Escherichia coli* ATCC 25922, and *Streptococcus agalactiae* ATCC 13813. The data were statistically analysed using R software and the detection limit (LOD) and sensitivity were determined. **Results.** After optimization, the developed RT-qPCR assay demonstrated in a partial validation a high analytical sensitivity, detecting as few as 100 RNA copies/reaction with consistent amplification across three replicates. The limit of detection (LOD) was established at 1.0×10^2 RNA copies for both genotypes in a dilution series of virus RNA. Standard curves based on Ct values versus $\log_{10}$ of RNA concentration yielded slopes of –3.44 (GI.1) and –3.38 (GI.2), corresponding to amplification efficiencies of 95.4% and 97.5%, respectively. The assay showed excellent linearity ($R^2 = 0.925$ for GI.1, $R^2 = 0.881$ for GI.2) and intra-assay variability (%CV) below 3.5% across all tested dilutions. No cross-reactivity was observed with rabbit myxomatosis virus and four bacterial pathogens. In a sample containing both genotypes, the assay successfully detected and differentiated both GI.1 and GI.2 targets. **Conclusions.** In this study, we designed a multiplex real-time RT-qPCR assay targeting the VP10 gene (ORF2) for the detection and differentiation of rabbit hemorrhagic disease virus (RHDV) genotypes GI.1 and GI.2 circulating in Ukraine. The assay demonstrated promising analytical performance, with an estimated limit of detection of 100 RNA copies/mL and no observed cross-reactivity with selected non-target pathogens. These results provide a partial validation of the method, indicating its potential applicability for laboratory diagnosis and epidemiological investigations.

However, several limitations must be acknowledged. The current validation was restricted to a limited number of samples and two genotypes, while GI.3, GI.4, GII.1, GII.2, and recombinant variants were not included. No internal amplification control was applied, and (inter-laboratory) comparison with the WOAH-recommended VP60-based RT-PCR has yet to be conducted. Future studies should complete the validation by expanding the range of tested genotypes, incorporating an internal control, and performing multi-center validation. Additional testing on portable diagnostic platforms and under field conditions will further determine the assay's suitability for routine veterinary practice. Pending such validation, the VP10-based multiplex RT-qPCR assay then may complement existing VP60-based diagnostics, or serve as a (preliminary) control of those classical diagnostics, when surveying RHDV in Ukraine.

Keywords: rabbit hemorrhagic disease virus, validation, analytical sensitivity, specificity, molecular detection, primers.

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INTRODUCTION

Rabbit hemorrhagic disease (RHD) is a highly contagious and often fatal viral disease of rabbits, first described in China in 1984 (Liu et al., 1984; Abrantes & Lopes, 2021). This disease is caused by rabbit hemorrhagic disease virus (RHDV), a non-enveloped icosahedral virus with a single-stranded RNA genome, approximately 7.5 kb in length and a sub-genomic RNA of 2.2 kb (Le Pendu et al., 2017; WOAH, 2023). The virus belongs to the genus *Lagovirus* of family *Caliciviridae* and the species *Lagovirus europaeus*. Lagoviruses are genotypically classified by the core capsid protein (VP60) (Le Pendu et al., 2017). The first outbreaks of RHDV (genotype GI.1) occurred in China in 1984 (Liu et al., 1984), after which the virus spread rapidly across Europe and other continents (Hu et al., 2021; O'Connor et al., 2022; Hu et al, 2023; Shah et al., 2023; Hu et al., 2025). GI.1 strains were also deliberately introduced in Australia and New Zealand for biological control of wild rabbit populations (O'Connor et al., 2022; Tu et al., 2022; Hall et al., 2024). In the middle of the 1990s, antigenic variants of RHDV were found (RHDVa or genotype GI.1a) (Capucci et al., 1998). In 2010, in France, RHDV2 (genotype GI.2) was first described (Le Gall-Reculé et al., 2013), and its mutation regions cover both non-structural (NS) and structural (S) genes. This genotype is very lethal, also to other lagomorphs (see below) and rapidly spread globally (Mahar et al., 2017; Mahar et al., 2021; Abade Dos Santos et al., 2022; Byrne et al., 2022; Calvete et al., 2022; Fresco-Taboada et al., 2022; Cooke, 2024; Sun et al., 2024). Lethality of RHDV GI.2 may reach 90.0% in populations of susceptible animals, which highlights the significance of this variant of the pathogen (Erfan & Shalaby, 2020). GI.2 was also detected in mountain hare (*Lepus timidus*) in Sweden (Neimanis et al., 2018) and a recombinant of GI.2 in Iberian hare (*L. granatensis*) in Spain (Velarde et al., 2021).

In Ukraine, RHDV remains endemic, with both GI.1 and GI.2 variants currently co-circulating. According to our earlier studies, GI.1 (including the RHDVa/GI.1a antigenic variant) was historically widespread, while GI.2 has been increasingly detected since 2021 (Mezhenskyi et al., 2023). Between 2021 and 2023, GI.2 was confirmed in outbreaks in central (Kyiv, Poltava), southern (Odesa, Mykolaiv, Kherson), and western (Lviv, Ternopil, Zakarpattia) regions, while GI.1 cases persisted mainly in eastern oblasts (Kharkiv, Dnipro) and sporadically in the north (Chernihiv, Zhytomyr) (Mezhenskyi et al., 2024). Overall, in 2021–2023, GI.2 accounted for approximately 70–75% of sequenced cases, reflecting its increasing dominance in the Ukrainian rabbit population, whereas GI.1 was identified in 25–30% of samples, particularly in older breeding farms with limited animal movement. These data indicate a shift in genotype prevalence in Ukraine similar to trends observed in other European countries, underscoring the need for differential diagnostic tools capable of detecting both genotypes.

The structural major capsid protein VP60 (encoded by ORF1) and the minor capsid protein VP10 (encoded by ORF2) are important antigens that influence receptor binding, virulence, and pathogenicity of RHDV (Mahar et al., 2021; Calvete et al., 2021). Most molecular diagnostic assays have traditionally targeted the VP60 gene, as it forms the basis for RHDV classification and typing (WOAH, 2023). However, alternative approaches have also been explored. Luo et al. (2019) developed an RT-qPCR assay targeting the VP10 gene, demonstrating that ORF2

may serve as a diagnostic marker, although its variability has also been reported (Shah et al., 2023).

Both pathogenic and non-pathogenic forms of RHDV have been discovered in recent years. Some studies demonstrate that pathogenic lagoviruses could appear either via direct evolution from the non-pathogenic ancestor, or via the transition between species (Esteves et al., 2015; Aguayo-Adán et al., 2021). Multiple mutational changes and recombination events in the RHDV genome were observed in different countries over the years and they play a significant role in the evolution, spreading, and genetic diversity of the RHDV virus (Lopes et al., 2015; Abrantes et al., 2020; Bębnowska et al., 2021; Fitzner et al., 2021; Asin et al., 2022; Chen et al, 2022; Pacioni et al., 2022; Cavadini et al., 2024; Peacock et al., 2024; Tokarz-Deptuła et al., 2024;).

Diagnosis of RHDV and its causal agent can be made on the basis of symptomatology, serology (mainly ELISA, using monoclonal antibodies) and molecular biological methods, mainly reverse-transcriptase (RT-) PCR (Niedźwiedzka-Rystwej et al., 2013; Dalton et al., 2018; Hall et al., 2018; Kwit & Rzeżutka, 2019; Fresco-Taboada et al., 2022; Korovin et al., 2024; WOAH, 2023 Standard for RHDV, https://www.woah.org/en/disease/rabbit-haemorrhagic-disease/ (last retrieved August 2025).

Multiplex PCR assays have been successfully applied for the discrimination of RHDV genotypes, including recombinant variants, which is of decisive significance for epidemiological supervision and outbreak management (Gall et al., 2007; Hall et al., 2018; Fitzner et al., 2021). However, there are still limitations regarding cross-reactivity, the emergence of recombinant strains that may not be detected by existing assays, and the need for continuous updating of standardized protocols under different field conditions (Kwit & Rzeżutka, 2019; Mahar et al., 2021; WOAH, 2023).

Reverse-transcriptase PCR in its different forms has advantages over traditional serological tests, such as the hemagglutination reaction and enzyme-linked immunosorbent assay (ELISA) due to higher sensitivity, specificity, and ability to directly detect viral RNA (Abrantes & Lopes, 2021). Platforms of RT-qPCR and RT-LAMP-CRISPR/Cas12a yield faster processing of results and lower requirements for equipment, which allows using them not only in laboratories, but, in case of RT-LAMP, even under field conditions (rabbit-breeding farms) (Wu et al., 2024). There are also ongoing studies aimed at simultaneous detection of multiple pathogens. For example, Tung et al. (2018) developed a DNA biochip hybridisation assay capable of detecting several bacterial and viral agents of veterinary importance, including *Pasteurella multocida*, *Escherichia coli*, and *Salmonella enterica*. Fresco-Taboada et al. (2022) reported a duplex lateral flow assay allowing the differential detection of RHDV genotypes GI.1 and GI.2.

As stated above, beyond the widely used VP60 target, the VP10 (ORF2) region has also been explored for RT-qPCR. Luo et al. (2019) reported a SYBR Green real-time RT-PCR targeting VP10 for detection of classical RHDV, demonstrating high analytical sensitivity and specificity on plasmid standards and selected non-target bacteria. However, their assay did not cross-react with GI.2 gene fragments, suggesting limited detection of this genotype.

Earlier Ukrainian protocols (Krytsia et al., 2022; Korovin et al., 2024) either lacked published primer sequences or showed limited specificity, restricting their use for routine diagnostics.

The integration of multiplex PCR with other diagnostic platforms remains poorly standardized, and the WOAH-recommended protocols are not consistently applied in practice. In addition, comparative studies between assays are still limited, which hampers the development of clear recommendations for routine application (Kwit & Rzezutka, 2019; Abrantes & Lopes, 2021). In this context, we designed and partially validated a VP10-based multiplex RT-qPCR assay capable of discriminating between RHDV genotypes GI.1 and GI.2 currently circulating in Ukraine. While our preliminary data demonstrate promising analytical sensitivity and specificity, further validation, including inter-laboratory testing and comparison with VP60-based assays, is required before its implementation in routine laboratory or field diagnostics. Recent evidence also indicates that the VP10 gene is subject to genetic variability (Shah et al., 2023), underscoring the need for continued assay optimization.

MATERIALS AND METHODS

Strains of the rabbit hemorrhagic disease virus (RHDV), maintained in the virus collection of the Institute of Veterinary Medicine of the National Academy of Agrarian Sciences of Ukraine (IVM NAAS), Kyiv, were used in testing. The following rabbit (pathological) material was used: RHDV G1.1 and

RHDV G1.2 positive and negative field material; material of a rabbit infected with a reference strain of RHDV GI.1 (strain BS-89) and of RHDV GI.2 (strain TA 14). Specificity of the assay was further tested using non-target pathogens: For specificity testing, in addition to RHDV isolates, non-target organisms were included: *Myxoma virus* (strain B-82, IVM NAAS collection), *Escherichia coli* ATCC 25922, *Pasteurella multocida* subsp. *multocida* ATCC 12945, *Staphylococcus aureus* subsp. *aureus* ATCC 25923 and *Streptococcus agalactiae* ATCC 13813. All RHDV-positive rabbit material from the IVM NAAS collection had previously been diagnosed by RT-PCR targeting the VP60 gene, according to guidelines by Krytsia et al., 2022 and WOAH, 2023, and the genotype (GI.1 or GI.2) was confirmed by the test kit INgezim® RHDV1/2 DIF CROM (R.17.RHD.K.42) (Mezhenskyi et al., 2023).

Oligonucleotide primers were developed for the ORF2 gene encoding the minor capsid protein VP10. This region has previously been proposed as an alternative diagnostic target (Luo et al., 2019), although genetic studies have also demonstrated that VP10, similar to VP60, is subject to recombination and variability (Shah et al., 2023). In contrast to the SYBR Green VP10 assay of Luo et al. (2019), we designed a probe-based multiplex RT-qPCR targeting VP10 with genotype-specific hydrolysis probes to discriminate GI.1 and GI.2 within a single reaction. Design choices were guided by in-silico analyses of GI.1 and GI.2 diversity and by the need to minimize false-negative results in the presence of emerging variants and recombinants. The probes were synthesized by Thermo Fisher Scientific (USA) at our request.

Multiple alignment of gene sequences was performed in CLUSTAL_X (http://clustalx.software.informer.com) using sequences of ORF2, encoding the minor capsid protein VP10, present in the full genome sequences of known strains of the RHDV, including reference strains and isolates, the sequences of which were added to the database after 2020.

A total of 38 full-genome sequences of RHDV genotype GI.1 and GI.2 of different geographic origin present in GenBank were analyzed:

Genotype GI.1, 16 sequences: China: MK814815.1, MK895974.1, AY269825.1, KJ814617.1, JN165233.1, DQ530363.1; France: LR862105.1; Germany: LR899177.1, LR899172.1, LR899169.1, LR899138.1, LR899174.1, EF558581.1; Iran: KT006727.1; Italy: OM372629.1; Ukraine: OP588107.1.

Genotype GI.2, 22 sequences: China: MT383749.1, OQ570963.1, OQ570964.1, MT737965.1, MT434995.1, MW974834.1, MT586027.1, MW178244.1, OK665346.1, OQ570961.1, MT495252.1, MT383748.1, MT472573.1, OQ570960.1, OQ570962.1, MT505389.1; Egypt: MZ913393.1; Germany: LR899146.1, MN901451.1; Iran: KT006723.1; Tunisia: MZ782086.1; Ukraine: MW460218.1.

After comparing the sequences *in silico*, we identified target sites for specific detection and constructed a set of oligonucleotide primers, which was checked for potential cross-reactivity using BLAST (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/BLAST), and for preliminary sensitivity and specificity, using multiplex RT-qPCR with specific and non-specific genetic material from the laboratory collection, mentioned above.

The list of primers and probes used is presented in *Table 1*.

Nucleic acid isolation was conducted using the IndiSpin® Pathogen Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNA concentration was determined using the Qubit RNA HS kit (Thermo Fisher Scientific, USA). Ten-fold se-

Table 1. Oligonucleotide primers and probes used for the detection of cDNA of RDHV, using our newly developed multiplex RT-qPCR

Name	Sequence (5′–3′)	Purpose	Length, bp	Tm, °C	GC, %	Fluorophore/ quencher
VP10-F	TGTCTGAATTTGTTGGACTAGGA	Forward primer (common)	23	59.8	39.0	—
VP10-R	TGGCCTTTAAAACCAACCCA	Reverse primer (common)	20	60.2	45.0	—
Probe-VP10-GI.1	CAGGTGCCAGCGTTTTGAGCA	Probe for GI.1	21	68.7	52.4	FAM-BHQ1
Probe-VP10-GI.2	CAGGTGCCAGTGTTTTGAGTA	Probe for GI.2	21	68.5	47.6	HEX-BHQ1

rial dilutions of viral RNA (ranging from 10^6 to 10^0 copies/reaction) were prepared in RNase-free water supplemented with carrier RNA. Each dilution was tested in triplicate by multiplex RT-qPCR. Calibration curves were generated by plotting the logarithm of the RNA copy number against the crossing point (Ct) values using the CFX Manager™ Software (Bio-Rad, USA). The number of RNA copies per reaction was calculated based on the RNA concentration (ng) and the molecular weight of the transcript, using the formula: RNA copy number = (amount of RNA in g)/(transcript length × 340 Da × 6.022×10^{23}). Amplification efficiency (E) was determined from the slope (k) of the regression line according to the equation: $E = [10^{-1/k} - 1] \times 100\%$.

For RT-qPCR we used the Invitrogen™ SuperScript™ III Platinum™ One-Step RT-qPCR Kit (Cat. No. 11732020), containing reverse transcriptase and fast start polymerase. The composition of the reaction mixture as per a volume of 25 µl is presented in *Table 2*.

To ensure high sensitivity and specificity, the RT-qPCR steps were optimized as indicated in *Table 3*.

Statistical analysis was performed using the R software package (R version 3.4.2, 2016). The results were considered statistically significant if $P < 0.05$. Standard deviation (SD) and coefficient of variation (CV) were calculated in accordance with ISO/IEC 17025:2018 requirements (ISO, 2018).

RESULTS

To detect the RHDV GI.1 and GI.2 genotypes, a specific primer set for RHDV was developed, targeting the ORF2 sequence coding for the VP10 minor capsid protein, namely VP10-F (5′-TGTCTG AATTTGTTGGACTAGGA-3′) and VP10-R (5′-TGG CCTTTAAAACCAACCCA-3′), which amplifies a fragment of 101 bp located within ORF2, see *Table 1*. The primer set demonstrated high specificity to the target (16 sequences of genotype GI.1) and no cross-reactions with 22 sequences of genotype GI.2 in the *in-silico* analysis (BLASTn).

Subsequently two genotype-specific probes were designed in order to differentiate between genotypes GI.1 and GI.2. Probe VP10-GI.1 (FAM-CAGGTGC-CAGCGTTTTGAGCA-BHQ1) completely corresponds to the homologous sequence in GI.1, whereas probe VP10-GI.2 (HEX-CAGGTGCCAGTGTTTT GAGTA-BHQ1) contains two single nucleotide replacements (SNP), ensuring the specificity to GI.2, also see *Table 1*. The evaluations *in silico* demonstrated a difference of more than 7°C in the melting temperature (ΔTm) between probes and non-relevant targets, which ensures high selectivity at the anneal-

Table 2. Composition of the multiplex RT-qPCR reaction mix

Component	Name or catalogue No.	Volume, µl	Final concentration
2× One-Step RT-qPCR Master Mix	Invitrogen	12.5	1×
Forward primer (10 µM)	VP10-F	0.8	400 nM
Reverse primer (10 µM)	VP10-R	0.8	400 nM
Probe (10 µM)	Probe-VP10-GI.1 (FAM)	0.5	200 nM
Probe (10 µM)	Probe-VP10-GI.2 (HEX)	0.5	200 nM
RNAse-free water	—	7.9	—
RNA-template (isolated RNA)	—	2.0	~10^2–10^3 copies
Total volume	—	25.0	—

Table 3. Cycling conditions of the multiplex RT-qPCR

No	Steps	Temperature, ºC	Time	Number of cycles
1	Reverse transcription	50	30 min	1
2	Initial DNA denaturation	95	10 min	1
3	DNA denaturation	95	20 s	40
	Primer annealing	60	20 s	
	Elongation	72	20 s	

ing temperature of 60°C. Both probes were tested also *in silico* (BLASTn) for the absence of cross-hybridization, and it was confirmed *in vitro* using the reference isolates RHDV GI.1 (strain BS-89) and RHDV GI.2 (strain TA 14), that no cross-reactivity occurred between the two genotypes.

The confidence interval for the analytical sensitivity of RHDV was 100% at the concentration of 1.0×10^6–1.0×10^2 RNA copies/ml. yielding an LOD of c.10_2 copies/mL of viral RNA in an RNA dilution series for RHVD GI.1 and GI.2 (*Table 4*). Each dilution was tested in triplicate by multiplex RT-qPCR. Calibration curves were generated by plotting Ct values against the logarithm of RNA copy number (see *Fig. 1*).

The results presented in *Table 4* demonstrate that the multiplex RT-qPCR assay reliably detected viral RNA dilutions in the range of 1.0×10^5 to 1.0×10^2 copies per reaction. Within this concentration range, all three replicates for both GI.1 and GI.2 were consistently positive, corresponding to 100% detection probability. At 1.0×10^1 copies per reaction, amplification was inconsistent, and below this level no amplification was observed. Therefore, the limit of detection (LOD) of the assay was defined as 1.0×10^2 RNA copies/mL for both genotypes.

To determine the efficiency of our multiplex RT-qPCR and the linearity of amplification, standard curves were built, based on the results of the reaction with the 10-fold serial dilutions of the RNA of the RHDV genotype GI.1 and GI.2, see *Fig. 1*. A high correlation level was obtained for both genotypes, which demonstrates good repeatability and amplification within the concentration range from 10^6 to 10^2 copies of RNA.

The amplification curves were used to build standard regression dependencies between the concentration logarithm ($\log_{10}$) and the average value of Ct. For genotype GI.1, the regression equation was as follows: $y = -3.44x + 41.37$, with the determination GI.1 the coefficient value of $R^2 = 0.925$ was obtained and for genotype GI.2 a value of $R^2 = = 0.881$was obtained. These values demonstrate high linearity and repeatability (intra-assay CV <3.5%) of the reaction within the dynamic range. The estimated efficiency of amplification was 95.4% for GI.1 and 97.5% for GI.2 (*Table 5*).

Table 4. Determination of detection limit (analytical sensitivity) and repeatability using a 10-fold dilution series of viral RNA of RHDV genotypes GI.1 (strain BS-89) and GI.2 (strain TA 14), prepared from liver tissues in RNase-free water supplemented with carrier RNA in three repeats

No	No. of RNA copies/mL of RHDV in dilution	Ct_1	Ct_2	Ct_3	Average for three repeats	SD	Repeatability (Intra-assay CV, %)
1. Results for RHDV GI.1							
1.1.	1.0×10^6	18.17	19.33	18.61	18.70	0.59	3.16
1.2.	1.0×10^5	21.22	20.33	20.14	20.56	0.57	2.79
1.3.	1.0×10^4	23.16	23.82	22.27	23.08	0.78	3.38
1.4.	1.0×10^3	26.82	25.18	26.34	26.11	0.83	3.17
1.5.	1.0×10^2	33.03	32.03	34.26	33.11	1.12	3.38
1.6.	1.0×10^1	32.16	34.77	ND	ND	ND	ND
1.6.	1.0×10^0	ND	ND	40.1	ND	ND	ND
2. Results for RHDV GI.2							
2.1.	1.0×10^6	19.32	19.18	19.30	19.27	0.07	0.36
2.2.	1.0×10^5	21.60	21.17	21.29	21.35	0.22	1.03
2.3.	1.0×10^4	23.14	22.77	23.01	22.97	0.19	0.83
2.4.	1.0×10^3	25.43	26.38	25.92	25.91	0.48	1.86
2.5.	1.0×10^2	34.21	34.06	33.46	33.91	0.39	1.15
2.6.	1.0×10^1	ND	ND	ND	ND	ND	ND
2.6.	1.0×10^0	ND	ND	ND	ND	ND	ND

Note: ND indicates that amplification was not detected in any of the triplicates.

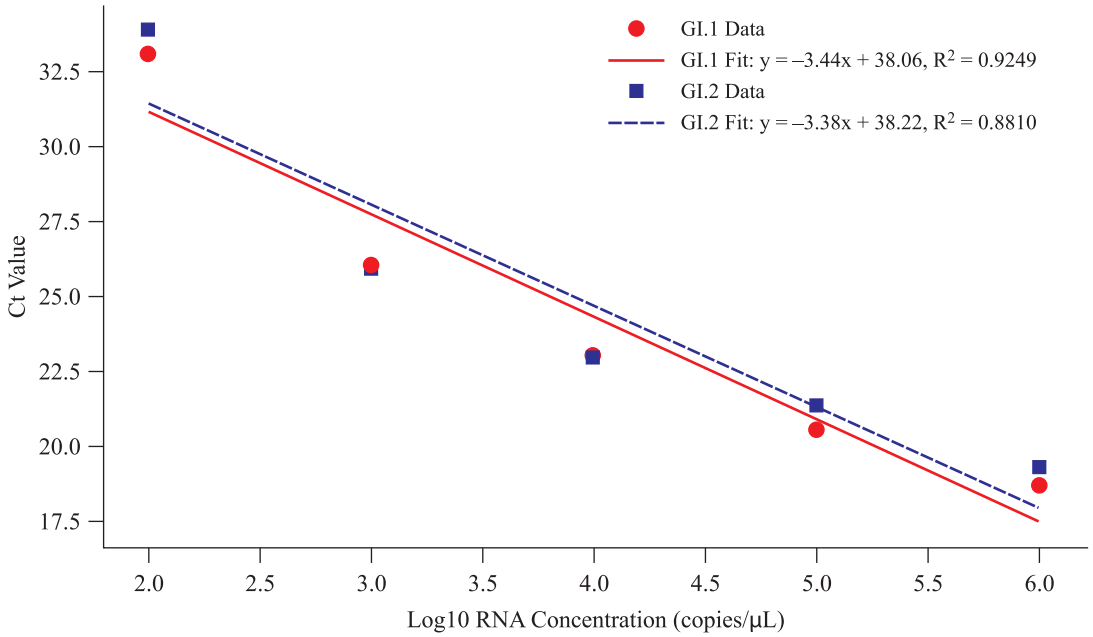

Fig. 1. Standard curves generated from Ct values plotted against the $\log_{10}$ of RNA copy numbers for RHDV GI.1 and GI.2. Dilution series corresponded to 1.0×10^6 to 1.0×10^2 RNA copies per reaction. Regression equations: GI.1: y = –3.44x + 41.37 (R^2 = 0.925); GI.2: y = –3.38x + 39.7 (R^2 = 0.881)

Table 5. The efficiency of the multiplex RT-qPCR reaction (by the standard curve Ct vs $\log_{10}$ (copies of the genome)

Genotype	Linear equation*	Slope (k)**	Efficiency, E (%)
RHDV GI.1	Ct = –3.44 × $\log_{10}$ (conc.) + 39.4	–3.44	95.4%
RHDV GI.2	Ct = –3.38 × $\log_{10}$ (conc.) + 39.7	–3.38	97.5%

* Linear equation: regression line obtained by plotting Ct values against the logarithm (base 10) of RNA copy number from the dilution series. The slope (k) was used to calculate amplification efficiency (E) according to the formula: $E = [10^{-1/k} – 1] \times 100\%$.
** Slope (k) is derived from the regression line.

The values for the curve slopes and efficiency values were within the acceptable intervals (from –3.1 to –3.9) and 95–110%, respectively, which indicated the absence of any inhibition or considerable losses during amplification. The results of determining the analytical specificity of the test for both RDHV genotype GI.1 and GI.2 are reported in *Table 6*.

Data, presented in *Table 6*, demonstrate, although for a limited number of samples and virus variants, that no false-positive or -negative results were obtained.

Our results represent a limited validation of the assay, demonstrating analytical sensitivity and specificity of the developed real-time RT-qPCR method for detection of RHDV genotype GI.1 and GI.2. The current study assessed intra-assay variability only. Inter-assay variability across different runs and thermocyclers remains to be evaluated. The assay demonstrated promising analytical sensitivity with an LOD of 100 copies, although broader validation is required.

DISCUSSION

To detect RHDV, the World Organisation for Animal Health (WOAH, formerly OIE) currently recommends a single-step RT-PCR assay targeting the VP60 gene (WOAH, 2023). The recommended primers amplify all known RHDV variants, including RHDV GI.2, using forward primer 5′-CCT-GTT-ACC-ATC-ACC-ATG-CC-3′ and reverse primer 5′-CAA-GTT-CCA-RTG-SCT-GTT-GCA-3′. For RHDV GI.2-specific detection, alternative primer pairs have been described, including 14U1/RVP60-L1, which amplify a 794 bp fragment in the C-terminal region of VP60

Table 6. Results of determining the specificity of the developed multiplex RT-qPCR method *in vitro*, using contaminated and non-contaminated material in three repeated PCR's, M ± m, n = 3

No	Investigated material	Ct for FAM (RHDV GI.1)	Ct for HEX (RHDV GI.2)
1	Rabbit pathological material, containing RHDV GI.1	19.80±0.13	ND
2	Rabbit material, not containing RHDV, negative control	ND	ND
3	Rabbit pathological material, containing RHDV GI.2	ND	26.58±0.14
4	Rabbit material, not containing RHDV, negative control	ND	ND
5	Rabbit pathological material, containing RHDV GI.1 (strain BS-89) + RHDV GI.12 (strain TA 14) (1:1)	22.22±0.39	31.53±0.16
6	Cell culture containing myxomatosis virus (*Myxoma virus*, strain B-82), at ~1.0×10^6 RNA copies/ml	ND	ND
7	Culture of the causal agent of rabbit staphylococcosis (*Staphylococcus aureus* subsp. *aureus* ATCC 25923), standardized to ~1.0×10^6 CFU/ml, suspended in phosphate-buffered saline (PBS).	ND	ND
8	Culture of the causal agent of rabbit streptococcosis (*Streptococcus agalactiae* ATCC 13813), standardized to ~1.0×10^6 CFU/ml, suspended in phosphate-buffered saline (PBS).	ND	ND
9	Culture of the causal agent of pasteurellosis of rabbits (*Pasteurella multocida* subsp. *multocida* ATCC 12945), standardized to ~1.0×10^6 CFU/ml, suspended in phosphate-buffered saline (PBS).	ND	ND
10	Culture of the causal agent of colibacillosis (*Escherichia coli*, ATCC 25922), standardized to ~1.0×10^6 CFU/ml, suspended in PBS.	ND	ND

Note: ND* — not detected.

(Le Gall-Reculé et al., 2013), and Fra109-F/Fra567-R, which amplify a 481 bp fragment in the N-terminal region of VP60 (Velarde et al., 2017).

However, several studies have demonstrated that frequent recombination events and high genetic variability in VP60 may reduce the sensitivity of assays targeting this region, particularly when detecting recombinant strains (Abrantes et al., 2020; Mahar et al., 2021; Shah et al., 2023). These challenges highlight the need for additional primer/probe sets targeting alternative genomic regions. In our work, we selected ORF2 (encoding VP10) as a complementary diagnostic target, based on earlier studies that explored its potential for RT-qPCR (Luo et al., 2019). While VP10 also exhibits variability, its use in multiplex format together with genotype-specific probes allowed us to achieve discrimination between GI.1 and GI.2 strains as they circulate in Ukraine.

However, accumulating evidence indicates that VP10 is also subject to substantial variability. Shah et al. (2023) reported significant amino acid drift and nucleotide variation within the VP10 region, with similarity levels between some strains dropping below 85%. Their analysis further demonstrated that VP10-based phylogeny is less consistent with whole-genome classification compared to VP60, and multiple recombination events have been identified within the VP60/VP10 coding region. Thus, while VP10 cannot be regarded as more conserved than VP60, it may still provide complementary information when used in multiplex assays. The multiplex format allows VP10-targeting probes to discriminate between GI.1 and GI.2, alongside the broader context of VP60-based diagnostics recommended by WOAH (2023).

Future multi-laboratory validation will be needed to confirm whether the VP10-targeted multiplex RT-qPCR maintains sensitivity and specificity across divergent lineages (e.g., classic GI.1, including RHDVa/GI.1a, versus contemporary GI.2 lineages co-circulating in Europe and North Africa) and representative

recombinants. When a full validation further confirms our results the assay could serve as a useful complement to WOAH-recommended VP60-based protocols for routine laboratory, field and farm diagnostics. This requirement is particularly important under conditions of co-circulation of evolutionarily distant isolates and periodic emergence of recombinants (Abrantes et al., 2020; Aguayo-Adán et al., 2021; Mahar et al., 2021). Notably, VP10 is not inherently more conserved than VP60 (Shah et al., 2023); our rationale for targeting ORF2 follows prior feasibility data for VP10-based RT-qPCR (Luo et al., 2019), with further optimisation and head-to-head comparison to VP60 assays recommended by WOAH (2023) still required.

ORF2, encoding VP10, contains genotype-associated single-nucleotide polymorphisms (SNPs) that can be exploited for differential probe design, enabling discrimination between GI.1 and GI.2 either in mono- or multiplex RT-qPCR formats. Our in-silico analysis confirmed an initial specificity of the selected probes, showing no cross-hybridization between genotypes, thus minimizing the risk of false positives or false negatives. This feature is particularly relevant given the continuous emergence of new variants and inter- or intragenotype recombinants (Luo et al., 2019; Mahar et al., 2021; Pacioni et al., 2022; Shah et al., 2023).

Recombination occurs not only between RHDV genotypes and variants but also between GI and GII European brown hare syndrome virus (EBSHV virus) (Perera et al., 2022). Therefore, when used in a routine setting a multiplex RT-qPCR, like the standard of WOAH and ours, requires regular monitoring. The tests used in molecular detection of RHDV infections may require regular review, and updates of primer sequences and negative results should be verified by another test (Kwit & Rzeżutka., 2019).

We obtained a high sensitivity in our *in vitro* dilution test of 100 copies/ml, although under field conditions, testing tissue extracts may yield a slightly lower sensitivity, expected to be 10^2~10^3 copies/reaction. This will be subject to further investigations and eventually a quantification limit has to be set (Hougs et al., 2017; Harcourt-Brown et al., 2020).

On the basis of an analysis of 38 complete ORF2 sequences available in GenBank (16 GI.1 and 22 GI.2) sequences present in GenBank, we choose sites that are less vulnerable to point mutation for our probes, to avoid any possibility of false negative results. This in relation to the above-mentioned frequent occurrence of mutations and recombination in *Lagovirus europaeus*.

Despite the wide use of PCR-based methods, there remain important gaps in the optimisation and validation of (multiplex) RT-qPCR assays for the simultaneous detection and differentiation of RHDV genotypes in clinical and field samples (Kwit & Rzeżutka, 2019). Recommendations by WOAH emphasise single-step RT-PCR targeting VP60 for routine diagnostics, partly because it reduces contamination risk compared with multi-step (e.g., nested) formats (WOAH, 2023). Moreover, several studies have shown that multiplex RT-PCR can differentiate among RHDV genotypes and reveal mixed infections or recombinant patterns within a single workflow, supporting its value for epidemiological surveillance when properly validated (Gall et al., 2007; Hall et al., 2018; Pacioni et al., 2022; Shi et al., 2024). In this context, our study contributes a VP10-targeted multiplex RT-qPCR with genotype-specific probes for GI.1 and GI.2 circulating in Ukraine, accompanied by partial analytical validation (standard curves, efficiency, LOD, and in-vitro specificity against non-target pathogens). Further head-to-head comparisons with WOAH-recommended VP60 assays and inter-laboratory studies are required before routine implementation (Luo et al., 2019; Mahar et al., 2021; Pacioni et al., 2022; Shah et al., 2023).

Our findings complement prior VP10-based work by Luo et al. (2019), who used a SYBR Green assay apparently tailored to classical RHDV (GI.1) and reported no cross-reaction with RHDV GI.2 fragments. By employing genotype-specific probes in a multiplex format, our assay provides differential detection of GI.1 and GI.2, addressing a practical need in regions where both genotypes co-circulate. Nonetheless, comprehensive validation — especially head-to-head against WOAH-recommended VP60 assays — remains necessary before routine implementation.

Rapid molecular diagnostics, including approaches that combine targeted amplification with sequencing, have been reported to provide results within a few hours in outbreak scenarios (Brinkmann et al., 2017; Fresco-Taboada et al., 2022; Zhang et al., 2023). In the near future, we intend to explore similar directions by testing our assay on portable diagnostic platforms such as the Oxford Nanopore MinION and field-deployable real-time PCR devices (e.g., Biomeme Franklin). In addition, we plan to investigate the po-

tential integration of isothermal amplification methods (e.g., RT-LAMP) to enable rapid diagnostics under field conditions.

CONCLUSIONS

In this study, we designed a multiplex real-time RT-qPCR assay targeting the VP10 gene (ORF2) for the detection and differentiation of rabbit hemorrhagic disease virus (RHDV) genotypes GI.1 and GI.2 circulating in Ukraine. The assay demonstrated promising analytical performance, with an estimated limit of detection of 100 RNA copies and no observed cross-reactivity with selected non-target pathogens. These results provide a partial validation of the method, indicating its potential applicability for laboratory diagnosis and epidemiological investigations.

However, several limitations must be acknowledged. The current validation was restricted to a limited number of samples and two genotypes, while GI.3, GI.4, GII.1, GII.2, and recombinant variants were not included. No internal amplification control was applied, and inter-laboratory comparison with the WOAH-recommended VP60-based RT-PCR has yet to be conducted.

Future studies should complete the validation of this VP-10 based PCR test by expanding the range of tested genotypes, incorporating an internal amplification control (IAC), and performing multi-laboratory validation. Additional testing on portable diagnostic platforms and under field conditions will further determine the assay's suitability for routine veterinary practice.

Pending such confirming final validation, the VP10-based multiplex RT-qPCR assay then may complement existing VP60-based diagnostics and may serve as a useful tool for epidemiological surveillance of RHDV in Ukraine.

Adherence to ethical principles. The study used only viral RNA without any association to the personal data; the methodological approaches corresponded to the requirements of standard IEC/ISO 17025:2018.

Conflict of interests. The authors claim the absence of any conflict of interests.

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ВИЯВЛЕННЯ ВІРУСУ ГЕМОРАГІЧНОЇ ХВОРОБИ КРОЛИКІВ (RHDV) ГЕНОТИПІВ GI.1 ТА GI.2 ЗА ДОПОМОГОЮ НОВОГО МУЛЬТИПЛЕКСНОГО RT-QPCR-ПРОТОКОЛУ В РЕАЛЬНОМУ ЧАСІ З ВИКОРИСТАННЯМ МІНОРНОГО КАПСИДНОГО БІЛКА VP10

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Мета. Метою цього дослідження було розроблення специфічного та чутливого мультиплексного методу зворотної транскрипції у реальному часі (RT-qPCR) для виявлення та диференціації генотипів GI.1 та GI.2 вірусу геморагічної хвороби кроликів (RHDV), що циркулюють в Україні, із використанням праймерів та проб, спрямованих на ген ORF2, який кодує мінорний капсидний білок VP10, та проведення початкової валідації цього методу. **Методи.** Аналіз було розроблено для ампліфікації консервативного фрагмента гена ORF2 довжиною 101 п.н., що кодує білок VP10, обраного як потенційно менш варіабельну альтернативу діагностичного маркеру VP60 (ген ORF1). Вирівнювання послідовностей здійснено з використанням 38 повних геномів ізолятів RHDV різного географічного походження з бази GenBank. Специфічні праймери та два генотип-специфічні гідролізні зонди (FAM для GI.1 та HEX для GI.2) були розроблені та частково валідовані як *in silico* (BLAST), так і *in vitro*. РНК екстрагували з шести зразків тканин (RHDV-позитивні та негативний контроль) і двох референтних штамів вірусу за допомогою набору IndiSpin Pathogen Kit. RT-qPCR виконували за одностадійним протоколом (деталі та послідовності наведені в основному тексті). Аналітичну чутливість і специфічність оцінювали за допомогою десятикратних серійних розведень РНК (від 10^6 до 10^0 копій/реакція). Для оцінки специфічності додатково тестували нецільові організми: вірус міксоматозу кроликів (штам B-82, колекція ІВМ НААН), *Staphylococcus aureus* ATCC 25923, *Pasteurella multocida* subsp. *multocida* ATCC 12945, *Escherichia coli* ATCC 25922 та *Streptococcus agalactiae* ATCC 13813. Статистичний аналіз проводили у програмному середовищі R. **Результати.** Оптимізований аналіз RT-qPCR

при частковій валідації продемонстрував високу аналітичну чутливість, виявляючи всього 100 копій РНК/реакцію з послідовним ампліфікацією у трьох повтореннях. Стандартні криві, засновані на значеннях Ct проти $\log_{10}$ концентрації РНК, дали нахили –3,44 (GI.1) і –3,38 (GI.2), що відповідає ефективності ампліфікації 95,4% і 97,5% відповідно. Метод продемонстрував високу лінійність (R^2 = 0,925 для GI.1, R^2 = 0,881 для GI.2) та низьку внутрішньоаналітичну варіабельність (%CV < 3,5%) для всіх протестованих розведень. Не було виявлено перехресної реактивності з іншими патогенами. У зразках, що містили обидва генотипи, аналіз успішно виявив і розрізнив обидва мішені GI.1 та GI.2. **Висновки.** Розроблено мультиплексний RT-qPCR-метод на основі VP10 (ген ORF2) для виявлення та диференціації генотипів GI.1 та GI.2 вірусу геморагічної хвороби кроликів (RHDV), що циркулюють в Україні. Аналіз продемонстрував високу аналітичну чутливість (щонайменш 100 копій РНК/реакцію), та відсутність перехресної реакції з вибраними нецільовими патогенами, що свідчить про його потенційне застосування для лабораторної діагностики та епідеміологічного нагляду. Водночас поточна валідація була обмежена невеликою кількістю зразків і двома генотипами, тоді як GI.3, GI.4, GII.1, GII.2 та рекомбінантні варіанти не були включені. Не застосовувався внутрішній контроль ампліфікації, а міжлабораторне порівняння з рекомендованим WOAH RT-PCR на основі VP60 ще не проводилося. Майбутні дослідження повинні враховувати ці обмеження шляхом розширення діапазону тестованих генотипів, включення внутрішнього контролю ампліфікації (IAC) та проведення багатоцентрової валідації. Додаткові випробування на портативних діагностичних платформах та в польових умовах дозволять остаточно визначити придатність тесту для рутинної ветеринарної практики. До проведення такої валідації мультиплексний RT-qPCR-тест на основі VP10 може доповнити існуючі діагностичні методи на основі VP60 та слугувати корисним інструментом для попереднього епідеміологічного нагляду за RHDV в Україні.

Ключові слова: вірус геморагічної хвороби кроликів, мультиплексна RT-qPCR, валідація, аналітична чутливість, специфічність, молекулярне виявлення, праймери.