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THE LABORATORY TESTING OF THE PCR-BASED PROTOCOL OF DETECTION OF THE RABBIT HAEMORRHAGIC DISEASE VIRUS RNA

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Abstract. *Rabbit Haemorrhagic Disease (RHD), also known as Rabbit Viral Haemorrhagic Disease (RVHD), is a highly contagious and often fatal viral disease that affects domestic and wild rabbits. It's caused by two related viruses: Rabbit Haemorrhagic Disease Virus (RHDV) and Rabbit Haemorrhagic Disease Virus 2 (RHDV2).*

The disease is endemic in many European, Asian and American countries, but the agent is still recognized as an emergent infection, associated with mass losses and extremely high mortality in rabbits of all breeds.

One Health Scientific and Research Institute, PSI in collaboration with SRI 'Veterinary Biotechnologies', LLC developed the in house PCR-based protocol for RHDV detection and RHDV-2 differentiation, that requires fast implementation. This diagnostics kit evaluation in described under OIE requirements with determination of the sensitivity, specificity, repeatability and domain-specificity. Detection kit is recommended for practical application.

Keywords: rabbit haemorrhagic disease virus, risk analysis, epidemiology, diagnostics, rabbits, PCR

Introduction. Rabbit Haemorrhagic Disease (RHD), also known as Rabbit Viral Haemorrhagic Disease (RVHD), is a highly contagious and often fatal viral disease in adult European rabbits (*Oryctolagus cuniculus*). This disease affects both wild and domestic rabbits. It's caused by two related viruses: Rabbit Haemorrhagic Disease Virus (RHDV) and Rabbit Haemorrhagic Disease Virus 2 (RHDV2). They belong to the family *Caliciviridae*, genus *Lagovirus* that causes rabbit haemorrhagic disease (RHD) in adult European rabbits (*Oryctolagus cuniculus*) (Abrantes, 2012, Tokarz-Deptuła, 2024).

RHD is primarily spread through direct contact with infected rabbits or their bodily fluids, such as saliva, urine, or feces. It can also be transmitted indirectly through contaminated objects or surfaces. The disease progresses rapidly, and affected rabbits may show symptoms such as fever, loss of appetite, lethargy, difficulty breathing, and bleeding from the nose or mouth. In some cases, rabbits may die suddenly without showing any symptoms (Prieto, 2000).

RHD particularly devastating in domestic rabbitries and can cause significant economic losses in commercial rabbit farming. Disease outbreaks can have a significant impact on rabbit populations, especially in areas where wild rabbits are abundant. The disease can spread quickly within a population, leading to high mortality rates (Hukowska-Szematowicz, 2012, Wang, 2012).

Vaccination is the most effective way to prevent RHD. Several vaccines are available, including ones specifically targeting RHDV and RHDV2 strains. Additionally, strict biosecurity measures, such as quarantining new rabbits and disinfecting equipment and facilities, can help prevent the spread of the disease (Mahar, 2018).

RHD has been reported in many parts of the world, including Europe, Asia, Australia, and the Americas. The emergence of RHDV2 in recent years has added complexity to disease control efforts, as this strain can affect previously vaccinated rabbits and has a wider host range (Wang, 2012, Mahar, 2018).

While RHD primarily affects rabbits, there is no evidence to suggest that it can infect humans or other animal species. However, strict biosecurity measures should still be followed

to prevent the potential spread of the virus to other susceptible animals (Nicholson, 2017, Pedler, 2016).

Overall, Rabbit Haemorrhagic Disease is a significant concern for rabbit owners, breeders, and wildlife managers due to its high mortality rate and ease of transmission. Vigilance, vaccination, and good biosecurity practices are essential for controlling and preventing outbreaks of this disease (Smertina, 2024).

Polymerase Chain Reaction (PCR) is a molecular biology technique used to amplify specific DNA/cDNA sequences, and it's a valuable tool in detecting Rabbit Haemorrhagic Disease Virus (RHDV) in infected rabbits. First, samples are collected from suspected cases of RHDV infection. These samples can include tissue samples (such as liver or spleen), blood, feces, or nasal swabs from affected rabbits (Aguayo-Adán, 2022).

PCR-based methods offer several advantages for RHDV detection, including high sensitivity, specificity, and rapid turnaround time. These assays are crucial for early diagnosis, surveillance, and control of RHDV outbreaks in rabbit populations (Hukowska-Szematowicz, 2023, Hrynkiewicz, 2022).

One Health Scientific and Research Institute, PSI in collaboration with SRI 'Veterinary Biotechnologies', LLC were developed the methodical approach for detection of the RNA of RHDV and RHDV-2 differentiation using Real-Time PCR due the reason of enhancement of RVHD control measures in Ukraine.

The aim of the presented research is to study sensitivity, specificity, repeatability and reproducibility of PCR-based protocol for detection of the RNA of RHDV and RHDV-2 differentiation using Real-Time PCR.

Materials and methods.

During laboratory testing trials evaluated appearance, activity, specificity, sensitivity PCR-based protocol for detection of the RNA of RHDV and RHDV-2 differentiation using Real-Time PCR and reproducibility.

The *in house* kit samples from batch 1, control evaluation 1 were used, produced in SRI 'Veterinary Biotechnologies' LLC, in April 2024.

The folowing samples panel has been used for specificity and sensityvity study of the test-system:

- reference panel cDNAs (n = 3) of the RHDV/RHDV-2, received from partner lab, and its 1 to 10, 1 to 100, 1 to 1000 and 1:10000 dilutions;
- RNA/cDNA samples isolated from the livers of infected rabbits (n = 7).
- RNA/cDNA samples isolated from the livers of the intact animals (n = 10).
- positive control of the amplification (PC);
- negative control of the amplification (NC);
- non-template control (NTC).

Following heterological templates were used to demonstrate the kit's species-specific specificity (genetic material of other cattle viruses):

- *Feline calicivirus virus* cDNA (vaccine);
- *Bovine herpesvirus* type 1 DNA (IBR virus);
- *Bovine diarrhea virus* cDNA (BVDV).

The polymerase chain reaction was managed under the recommended conditions described in the PCR-based protocol for detection of the RNA of RHDV and RHDV-2 differentiation Using Real-Time PCR. All testing was done without changes of the recommended features of reaction (table 1). To estimate the repeatability of testing this has been performed tree times.

Table 1

Amplification cycle for RHDV/RHDV-2 RNAs detection

Stage	Temperature	Duration	Step	Number of cycles
1	50ºC	10 min.	Reverse transcription	1
2	94ºC	3 min.	Activation of the DNA-polymerase	1
3	94ºC	10 sec	DNA denaturation	45
4	58ºc	45 sec	Primer annealing/ elongation	

Results. Tests were conducted to verify the commission sensitivity and specificity of the test system and reproducibility obtained when using the results.

At the beginning of the commission were checked for completeness of PCR-based laboratory in house test-systems. All components necessary for operation and instruction were available.

In assessing the appearance found that the test system consists of the following components:
RNA extraction kit (kit # 1):
- «extraction buffer» (lysis bufer) – 1 flack – 15 (30) ml, transparent non-coloured liquid;
- sorbent solution – 1 (2) tube - 1,5 ml, opalescentic fluid with white color;
- «Washing buffer» – 1 flack – 50 (100) ml, transparent non-coloured liquid;
- «Washing buffer ethanol» - 1 flack – 50 (100) ml, transparent non-coloured liquid;
- «solution 4» for the final washing – 1 flack – 30 (60) ml, transparent non-coloured liquid;
- TE-buffer – 1 flack – 20 (40) ml, transparent non-coloured liquid.

Kit for PCR amplification (kit # 2):
- «RT-One Step PCR MasterMix» – 1 (2) tubes, 0,5 ml, transparent non-coloured liquid;
- SYBR Green solution – 1 tube, 0,05 or 0,1 ml, transparent light green liquid;
- primer solution (20 pM/µl) – 1 tube of each (4 vials, 2 – for RHDV detection and 2 for RHDV-2 differentiation), 0,125 (0,25) ml, transparent non-coloured liquid;
- deionized water – 1 (2) tubes – 1,25 ml, transparent non-coloured liquid;
- positive control template (for 5 or 10 reactions) – 1 tube, 0,05 – 0,1 ml, transparent non-coloured liquid.

The PCR master mix was prepared using number of samples amount plus one under proportion per sample:

п/п	**Component**	**Final concentration**	**1 x for reaction (µl)**
1	Water for PCR		**6,7**
2	RT-PCR Master Mix- Path ID	**1X**	**9**
3	SYBR Green	**n/a**	**1**
4	Primer **RHDV_F (RHDV-2_F)** (20 pM/ µl)	**400nM**	**0,4**
5	Primer **RHDV_R (RHDV-2_R)** (20 pM/ µl)	**400nM**	**0,4**
	DNA (template or control)		**2**

The results of the sensitivity testing is the ability to identify all encrypted obviously positive samples, it was found that the test system is able to detect RNA of the RHDV and RHDV-2 virus in particular in both positive reference material samples, including their dilutions 1 to 10 – 1 to 10000 with Ct value 14.6-22.2. This is equal to the minimum titre of virus in the rabbit organism, we recorded an infected animal (in the liver and blood samples) in our previous research.

The specificity of the test system proved amplicon in the absence of any size of Ct value or Ct value over 38 in samples of rabbit origin tissues from non-infected animals.

In addition, primers designed didn't hybridized with DNA samples from other viruses, including DNA extracts from *Bovine herpesvirus* of types 1, and cDNAs of *Feline calicivirus* and *BVDV* (Table. 2).

It was also marked by complete coincidence test results developed RHDV /RHDV-2 detection protocol in three repetitions under similar conditions and using different Thermocyclers same type.

Table 2

Testing results for PCR-based protocol for detection of the RNA of RHDV using Real-Time PCR with the panels of positive, negative and heterogenic samples

#	Material	1st repeat result	2nd repeat result	3rd repeat result
1.	*Sample 1*	Ct 14.6	Ct 14.8	Ct 14.6
2.	*Sample 1 1:10*	Ct 18.2	Ct 18.9	Ct 20.9
3.	*Sample 1 1:100*	Ct 22.4	Ct 21.1	Ct 22.2
4.	*Sample 1 1:1000*	Ct 24.3	Ct 24.4	Ct 24.9
5.	*Sample 1 1:10000*	Ct 26.2	Ct 26.1	Ct 26.0
6.	*Sample 2*	Ct 16.2	Ct 16.4	Ct 17.2
7.	*Sample 2 1:10*	Ct 18.7	Ct 18.8	Ct 18.7
8.	*Sample 2 1:100*	Ct 21.2	Ct 21.4	Ct 21.2
9.	*Sample 2 1:1000*	Ct 24.5	Ct 23.9	Ct 24.1
10.	*Sample 2 1:10000*	Ct 27.2	Ct 27.2	Ct 27.1
11.	*Sample3*	Ct 18.6	Ct 18.8	Ct 18.9
12.	*Sample 3 1:10*	Ct 19.7	Ct 19.6	Ct 19.8
13.	*Sample 3 1:100*	Ct 22.4	Ct 22.3	Ct 22.6
14.	*Sample 3 1:1000*	Ct 25.3	Ct 25.2	Ct 24.9
15.	*Sample 3 1:10000*	Ct 27.2	Ct 26.7	Ct 26.7
16.	*Liver 1*	Ct 19.7	Ct 19.6	Ct 19.8
17.	*Liver 2*	Ct 14.2	Ct 14.8	Ct 14.4
18.	*Liver 3*	Ct 18.6	Ct 18.8	Ct 18.9
19.	*Liver 4*	Ct 18.2	Ct 18.4	Ct 18.3
20.	*Liver 5*	Ct 17.2	Ct 17.2	Ct 17.6
21.	*Liver 6*	Ct 18.4	Ct 18.2	Ct 18.5
22.	*Liver 7*	Ct 16.4	Ct 16.4	Ct 16.5
23.	*Liver (intact) 1*	N/d	N/d	N/d
24.	*Liver (intact) 2*	N/d	N/d	N/d
25.	*Liver (intact) 3*	N/d	N/d	N/d
26.	*Liver (intact) 4*	N/d	N/d	N/d
27.	*Liver (intact) 5*	N/d	N/d	N/d
28.	*Liver (intact) 6*	N/d	N/d	N/d
29.	*Liver (intact) 7*	N/d	N/d	N/d
30.	*Liver (intact) 8*	N/d	N/d	N/d
31.	*Liver (intact) 9*	N/d	N/d	N/d
32.	*Liver (intact) 10*	N/d	N/d	N/d
33.	IBRV	N/d	N/d	N/d
34.	FCV	N/d	Ct 42.0/ N/d	N/d
35.	BVDV	N/d	N/d	N/d

Amplification of RHDV-2 RNA demonstrated RNA presence in samples 2, 4, and 6 as well as in sample 1 of the reference panel. The Ct values for RHDV-2 amplification was observed on the levels 14.9 to 26.2.

Conclusions: 1. Specificity of the PCR-based protocol for detection of the RNA of RHDV detection using Real-Time PCR has the appropriate level of sensitivity and specificity, aligned with the detection of RHDV/RHDV-2 RNAs in clinical samples and their 1:10 to 1: 10000 dilutions, in Ct values of 14.6-22.2 (detection), and 14.9 to 26.2 (differentiation) with its absence of Ct value over 38 in the "negative samples". The developed kit doesn't demonstrate the false positive reactions with heterogenic DNA and RNA specimens, such as BHV-1, BVDV, and FCV.

2. The sensitivity of the system can be considered satisfactory, since positive results were positive for samples with low concentrations of specific RNA of RHDV/RHDV-2.

3. Reproducibility and repeatability of PCR-based protocol for detection of the RNA of RHDV detection using Real-Time PCR has the appropriate level, in all three repetitions differences were observed results.

The perspectives for further application of the results. As the laboratory testing of PCR-based protocol for detection of the RNA of RHDV detection using Real-Time PCR developed by One Health Scientific and Research Institute, PSI in collaboration with SRI 'Veterinary Biotechnologies', LLC indicators of quality, such as sensitivity, specificity and reproducibility fit the requirements. In this regard, this protocol can be recommended for interestablishmental testing and using in practice of surveillance and diagnostics of RVHD.

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ЛАБОРАТОРНА АПРОБАЦІЯ ПРОТОКОЛУ ПЛР-ДЕТЕКЦІЇ РНК ВІРУСУ ГЕМОРАГІЧНОЇ ХВОРОБИ КРОЛІВ

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***Резюме**. Геморагічна хвороба кролів (ГХК), також відома як вірусна геморагічна хвороба кролів (ВГХК), є висококонтагіозним і часто смертельним вірусним захворюванням, що вражає домашніх і диких кролів. Викликається двома спорідненими вірусами: Вірус геморагічної хвороби кроликів (RHDV) та Вірус геморагічної хвороби кроликів 2 (RHDV2).*

Захворювання є ендемічним у багатьох країнах Європи, Азії та Америки, але збудник все ще визнаний як емерджентна інфекція, пов'язана з масовими втратами та надзвичайно високою смертністю серед кролів усіх порід.

Науково-дослідний інститут єдиного здоров'я у співпраці з ТОВ "НДП "Ветеринарні біотехнології" розробив власний протокол на основі ПЛР для виявлення RHDV та диференціації RHDV-2, який потребує швидкого впровадження. Оцінка цього діагностичного набору описана відповідно до вимог МЕБ з визначенням чутливості, специфічності, повторюваності та доменної специфічності. Набір рекомендовано для практичного застосування.

Ключові слова: вірус геморагічної хвороби кролів, аналіз ризику, епідеміологія, діагностика, кролі, ПЛР

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