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*Original article*

# Real time PCR detection of rabbit haemorrhagic disease virus in rabbits infected with different European strains of RHDV

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## Abstract

The paper concerns the use of a novel, very effective diagnostic method, a real-time PCR for diagnosis of a viral agent causing viral haemorrhagic disease in rabbits – RHDV. Until now, the method was widely used for detecting many different viruses, both DNA, and RNA, but as far as RHDV is concerned, there are not many records of such use. This study aimed at the detection of 17 different strains from different European regions, differing in biological features and mortality. The study confirmed that real-time PCR is an applicable and effective method for diagnosis of RHDV, irrespective of the stains' features.

**Key words**: rabbit haemorrhagic disease, real-time PCR, viral diagnostics

## Introduction

In the 21st century, molecular biology methods are an important element in viral diagnostics. Apart from the classic PCR method, there are its variations and forms which significantly improve its specificity and sensitivity (Kubista et al. 2006, Watzinger et al. 2006). Among such methods, there is real-time PCR, namely amplification improved by the analysis of real-time growth in the product volume. A unique advantage of this method is its effectiveness, allowing for capturing very small volumes of virus copies, often unobtainable in regular PCR (Kubista et al. 2006, Watzinger et al. 2006). It must be stressed that in this technique, the determinant of the matrix DNA volume is the number of cycles after which reaction kinetics enters the phase of logarithmic growth in the product volume. The most frequent, and at the same time the easiest, real-time PCR method is to mark DNA with SYBR Green I fluorochrome which, by binding to double-stranded DNA, emits light. Some inconvenience of this system is the fact that this fluorochrome non-specifically binds to double-stranded DNA, yet in order to exclude the possibility of wrong interpretation, the analysis of product melting curves must be performed (Kubista et al. 2006, Watzinger et al. 2006, Lipiński et al. 2008). Real-time PCR method, as an element of viral diagnostics, was applied to assessment of viruses of DNA from the *Parvoviridae* (Dzieciątkowski et al. 2007), *Herpesviridae* (Grabarczyk et al. 2003, Radkowski et al. 2005, Dzieciątkowski et al. 2008), *Poxviridae* (Bacławski

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Table 1. RHDV stains used in the study, together with their biologic feature (haemagglutination, antigenic variants), country and date of origin and mortality caused in rabbits.

| RHDV strain | Haemagglutination | Antigenic variant | Country of origin | Date of origin | Mortality in 24 h |
|---|---|---|---|---|---|
| BS89 | HA+ | no | Italy | 1989 | 70% |
| Vt97 | HA+ | yes | Italy | 1997 | 30% |
| Pv97 | HA- | yes | Italy | 1997 | 90% |
| Hagenow | HA+/- | no | Germany | 1990 | 100% |
| Frankfurt | HA- | no | Germany | 1996 | 100% |
| Triptis | HA+ | yes | Germany | 1996 | 90% |
| Hartmannsdorf | HA+ | yes | Germany | 1996 | 90% |
| Rainham | HA- | no | England | 1993 | 90% |
| Asturias | HA- | no | Spain | 1996 | 90% |
| 9905 | HA- | yes | France | 1999 | 100% |
| 05-01 | HA+ | no | France | 2005 | 60% |
| 24V/89 | HA+ | no | Hungary | 1989 | 50% |
| 1447V/9 | HA+ | no | Hungary | 1996 | 12,5% |
| 72V/2003 | HA+ | yes | Hungary | 2003 | 0% |
| V-412 | HA+ | no | Czech Republik | 1989 | 40% |
| 237/04 | HA+ | no | Austria | 2004 | 100% |
| 01/04 | HA+ | no | Austria | 2004 | 100% |

2006) and *Hepadnaviridae* (Scaramozzimo et al. 2007) families, and RNA from the *Flaviviridae* (Bae et al. 2003, Pejsak et al. 2006, Gurukumar et al. 2009), *Retroviridae* (Kuźmiak et al. 2006) and *Caliciviridae* (Nowaczyk 2007, Fitzner et al. 2011, Teixeira et al. 2011) families. Within the last family, it was used for assessment of the volume of viral copies in the peripheral blood of rabbits infected with EBHS (European Brown Hare Syndrome), which is a pathogenic factor for hares (Nowaczyk 2007). Particularly interesting are the studies on the use of real-time PCR method with another virus from the *Caliciviridae* family – the RHD virus which causes rabbit plague, both among wild and farm rabbits (Niedźwiedzka 2008, Tokarz-Deptuła 2009). Since it is a virus that so far has not been proliferated *in vitro*, and hence its quick diagnostics is rendered difficult (Niedźwiedzka 2008, Tokarz-Deptuła 2009), this molecular biology method is becoming increasingly important, as other previously used methods for diagnostics of the virus, namely serological methods and electron microscopy, are insufficient in this respect. Research performed by Teixeira et al. (2011), referred to the use of a new method for isolation of the RHD virus from liver tissue, namely centrifugation at high rpm in iodixanol gradient, which can be very useful for further "processing", e.g. using real-time PCR. Also Fitzner et al. (2011) applied the real-time PCR method for assessment of 26 strains of the RHD virus (KGM 1988, SGM 1988, PD 1989, BLA 1994, MAL 1994, PRB 1995, BDG 1/1996, BDG 2/1997, BDG 3/1997, BDG 4/1998, GSK 1998, PIA 1999, POZ 199, ZD0 2000, SIZ 202, ZDU 2003, OPO 2004, GRZ 2004, ROK 2004, CB 2005, KRY 2005, ZKA 2005, DCE 2006, V-351, Frankfurt, PLF 83/92-353), in which not only the presence of RHD alone was assessed in particular liver samples. It was also shown that the limit of detection of viral RNA extracted from livers is at the $10^{-7}$ dilution.

The aim of the study was to qualitatively determine the occurrence of the RHD virus in liver cells of rabbits infected with seventeen strains of the RHD virus originating from various European countries, which differed not only in biological properties, such as haemagglutination capacity or antigenic variants, but also in pathogenicity (Table 1).

## Materials and Methods

The study involved liver tissue originating from rabbits which had died as a result of intramuscular infection with 17 analysed strains of the RHD virus (Table 1). Strains for animal infection were prepared as 20% liver homogenate cleared by centrifugation at 3000 rpm, 10% chloroforming for 60 minutes and

centrifugation again, and then suspension in glycerol in a 1:1 proportion (Niedźwiedzka-Rystwej and Deptuła 2010). Each virus strain used in the study for experimental infection came from an animal that had died of natural causes in its country of origin. All the samples prepared had the same number of viral particles specified by density suspended in caesium chloride, within the range of 1.34 g/dm$^3$ (Niedźwiedzka-Rystwej and Deptuła 2010).

After animal death, which occurred, depending on the strain of RHD virus administered, between 24 and 36 h from infection, liver tissue was collected *post mortem*. Next, it was made into a homogenate at a ratio of 1:3 with PBS, and from tissue prepared in this manner genetic material was isolated using an A&A Biotechnology isolation kit (DNA Gdańsk) according to the manufacturer's instructions. A complementary strand of nucleic acid (cDNA) was then obtained by reverse transcription reaction on the matrix of viral RNA, followed by real-time PCR reaction, using the conditions as described previously (Niedźwiedzka-Rystwej and Deptuła 2012). During the reaction, the level of stimulated fluorescence was continuously analysed, and melting curve analysis was performed for the purpose of determining specificity of the reaction (Niedźwiedzka 2008).

## Results

As a positive result (indicating the presence of the RHD virus in the analysed liver tissue), exponential growth in fluorescence was adopted (Fig. 1), whereas specificity of amplified products was confirmed with melting curves (Fig. 2). Table 2 additionally presents product melting temperature (Tm) and the number of reaction cycle where the sample's fluorescence entered the phase of logarithmic growth (Ct).

As indicated by the results obtained (Table 2, Figs. 1, 2), all the attempts confirmed the presence of the RHD virus in the analysed material with high resolution. It is also worth noting, that the Ct value, which falls before cycle 20, also confirms the high efficiency of the reaction. Also, the melting temperature test was measured to eliminate the possibility of non-specific product formation as a result of impact from SYBRGreen fluorescent colorant and, as shown, the melting temperature is very close in all samples, differing only by a maximum of 0.42°C. This fact also confirms high reaction specificity and proves the origin of the samples. The latter was also monitored by the selection of the primers, which were proposed on the basis of the conservative section of the genome of the RHD virus, occurring in region A of the VP60 polyprotein, namely the main structural protein of the virus. This fact gave a high confidence of the obtained product being RHDV, irrespective of differences in the strains' biology.

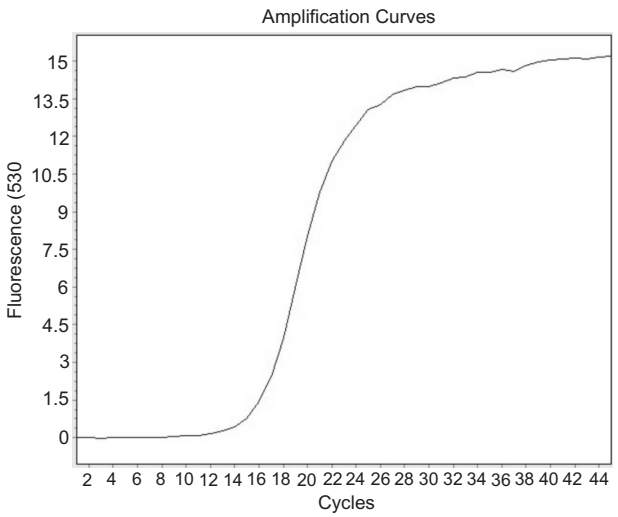


Fig. 1. Exemplary result of real-time PCR obtained for RHDV strain.

Table 2. Results obtained by detection with Real-time PCR of the liver samples in the direction of RHDV.

| RHDV stain | Result | Tm (°C) | Ct |
|---|---|---|---|
| BS89 | + | 87.54 | 10 |
| Vt97 | + | 87.31 | 15 |
| Pv97 | + | 87.26 | 16 |
| Hagenow | + | 87.53 | 11 |
| Frankfurt | + | 87.62 | 16 |
| Triptis | + | 87.35 | 14 |
| Hartmannsdorf | + | 87.29 | 12 |
| Rainham | + | 87.63 | 12 |
| Asturias | + | 87.61 | 13 |
| 9905 | + | 87.23 | 17 |
| 05-01 | + | 87.65 | 12 |
| 24V/89 | + | 87.26 | 16 |
| 1447V/9 | + | 87.54 | 12 |
| 72V/2003 | + | 87.35 | 16 |
| V-412 | + | 87.55 | 10 |
| 237/04 | + | 87.53 | 13 |
| 01/04 | + | 87.50 | 14 |

Legend: Tm – melting temperature; Ct – cycle threshold

## Discussion

Positive results, i.e. logarithmic growth of the fluoresce in the sample, were obtained for seventeen

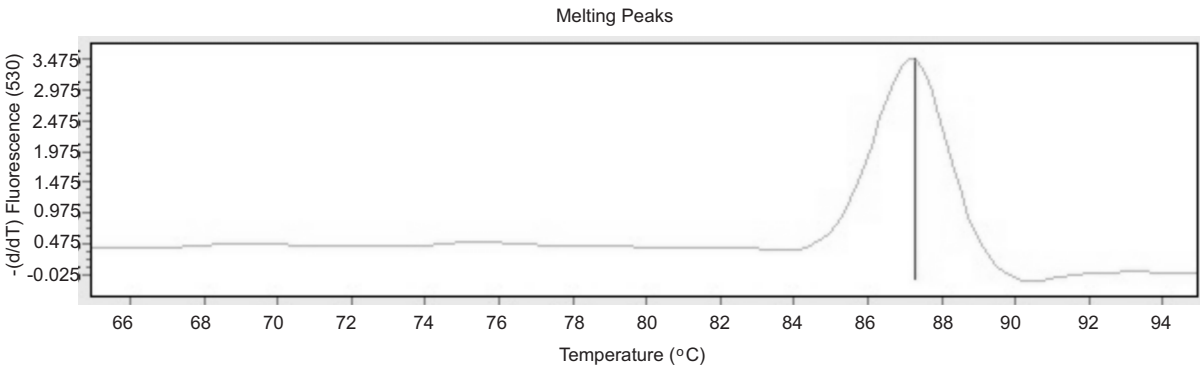


Fig. 2. Exemplary analysis of melting curves and temperature for RHDV strain.

different strains of RHDV. Since all the examined samples came from rabbits experimentally infected with RHDV, we could suppose that the mortality of the animals was the result of rabbit haemorrhagic disease. The positive result mentioned earlier, also confirms this. Thus the optimalisation and validation of the method was appropriate as it confirms the clinical observations (mortality of infected animals). Firstly, the conservative region primers, which were also applied in the study by Fitzner et al. (2011), permitted detection of all analysed strains of the RHD virus, and the result was not affected by differences recorded among the seventeen analysed strains of the RHD virus as regards their biological properties, such as haemagglutination capacity or formation of antigen variants, and time and place of isolation of such strains. Furthermore, various pathogenicity, measured using the mortality percentage of rabbits infected with the analysed strains, also did not affect the positive result of the real-time PCR reaction, which points to the fact that the method is unusually sensitive, and is not dependent on the volume of material to be detected, in this case the number of viral particles in the liver.

It is also worth noting that the method of isolation of the virus from the liver tissue in caesium chloride gradient, as used in the present study, is effective, while an alternative method may be the method of liver tissue isolation in iodixanol gradient, as indicated by Teixeira et al. (2011).

On the basis of the results obtained, and as indicated by literature, the real-time PCR method can be applied for assessment of liver samples from rabbits infected with the RHD virus regardless of their biological properties, time and place of isolation, and mortality among the infected animals. The technique is highly specific and efficient, and also allows significant shortening of the reaction time due to elimination of the post-amplification processing phase. It is also worth stressing the advantage of the method when using Light Cycler 1.5 equipment which requires the use of capillaries, which minimises the risk of contamination, so important for effectiveness of the reaction. To conclude, the real-time PCR method is very useful for the assessment of rabbit infection with the RHD virus and can be competitive with the electron microscopy and serological methods which are generally used in the diagnostics of this virus which causes a dangerous disease in laboratory and farmed rabbits.

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