Optimization of a real time PCR for the detection of rabbit haemorrhagic disease virus in infected rabbits from Azores

Optimização de um PCR em tempo real para deteção do vírus da doença hemorrágica do coelho nos Açores

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Summary: Some cases of Rabbit Haemorragic Disease (RHD) have been reported in the Azores and do the correct detection of agent pathogenic to this disease is important. Consequently, it was necessary to develop an in-house assay, which needed an adequate optimization and validation before be introduced in routine diagnostics. The aim of this study was to optimize the identification of the virus of haemorrhagic disease by RT-qPCR in order to use this technique in routine of Veterinary Regional Laboratory (LRV) of Azorean Government. The optimization of a RT-qPCR method for the rapid detection of the rabbit haemorrhagic disease virus (RHDV) was described using repeatability, reproducibility, specificity, sensitivity or detection limit. A primer set that targets 3’ part of VP60 gene and a Taq Man probe specific for the conserved region in RHDV genome was used in the method. The assay is able to detect genetic material in rabbits infected with classical RHDV and RHDVa. The specificity was 100% and the lowest limit of detection for series of dilution of a RHDV RNA-positive sample was found in 10^-5 dilution, to Ct=32. The hallmarks for an optimized RT-qPCR assay were reached, the reaction efficiency found was 94.5%, the R^2 value was 0.998 and consistency across samples (reproducibility) and replicates reactions (repeatability) was obtained. In conclusion, the studied method detects the RHDV and will be used in the routine of LRV. The replication of experiment by others veterinary laboratories is possible. Furthermore is a sensitive and reliable technique to RHD diagnosis.

Keywords: RHDV, rabbit, RT-qPCR, optimization.

Resumo: Alguns casos de doença hemorrágica do coelho (RHD) foram relatados nos Açores e fazer a detecção correta do agente patogênico causador desta doença torna-se importante. Por isso, foi necessário o desenvolvimento de um ensaio in-house, que precisava de uma otimização e validação adequadas antes de ser introduzido no diagnóstico de rotina. O objetivo deste estudo foi otimizar a identificação do vírus da doença hemorrágica por RT-qPCR, a fim de se utilizar esta técnica na rotina do Laboratório Regional de Veterinária (LRV) do Governo dos Açores. A otimização de um método RT-qPCR para a detecção rápida do vírus da doença hemorrágica dos coelhos (RHDV) foi descrita utilizando a repetibilidade, reproducibilidade, a especificidade, a sensibilidade ou o limite de detecção. Foram utilizados um conjunto de primers que tem como alvo a parte 3’ do gene VP60 e uma sonda específica de TaqMan da região conservada do genoma de RHDV. O ensaio utilizado é capaz de detetar material genético em coelhos infectados com a estirpe clássica de RHDV e RHDVa. A especificidade encontrada foi de 100% e o limite de detecção, para uma série de diluições de uma amostra de RNA positiva ao RHDV, foi encontrado na diluição 10^-5 com um Ct = 32. Foram alcançados todos os critérios necessários para a otimização do método RT-qPCR, designadamente a eficiência da reação foi de 94,5%, o valor de R^2 foi 0,998, obteve-se uma boa uniformidade entre as amostras ensaiadas (reproducibilidade) e entre réplicas das amostras (repetibilidade). Em conclusão, o método estudado permitiu a detecção de RHDV e vai ser utilizado na rotina do LRV. A replicação da experiência por outros laboratórios veterinários é possível. Além disso é uma técnica sensível e confiável para diagnóstico da RHD.

Palavras-chave: RHDV, coelho, RT-qPCR, otimização.

Introduction

Rabbit haemorrhagic disease (RHD) is caused by a non-enveloped ribonucleic acid (RNA) virus (OIE, 2010) that belongs to the genus Lagovirus and Caliciviridae family (Parra and Prieto, 1990; OIE, 2010). It is a virus constituted by a single stranded positive-sense genome of 7.5 Kb (Fitzner et al., 2011), with a capsid composed mostly of a single structural protein of 60 kDa in size (VP60) (Asgari et al., 1998) that causes a high contagious and lethal infection of wild and domestic rabbits of the species Oryctolagus cuniculus (Gall et al., 2007, Abrantes et al., 2012; Esteves et al., 2014; Duarte et al., 2014).

Transmission of RHDV may occurs through direct contact with an infected animal, since infected rabbits may shed viral particles in their secretions and excretion, or indirectly by means of fomites-contaminated food, bedding, water, clothing, cages and equipment or vector-borne transmission by scavenging mammals, birds and insects. The possible routes for transmission of the disease are the oral, nasal, conjunctival and...
Parenteral, blood-feeding insects have also been shown to be efficient mechanical vectors (Abrantes et al., 2012). Flies and other insects are the example of very efficient mechanical vectors (Asgari et al., 1998). In natural infections, the fecal-oral route is considered the preferential mode of transmission. In the field, carcasses of RHDV-infected rabbits may be the major source for viral spreading since the virus seems to be highly resistant and stable when exposed to harsh environmental conditions. Indeed, carcasses of RHDV-infected rabbits exposed at environmental conditions have been found to contain viable viral particles for up to three months (Abrantes et al., 2012). It was recently shown if dogs consume contaminated corpses can spread the virus through their feces and contaminate further through casual contact with other rabbits. The virus is not destroyed by freezing and contaminated meat can be transported one country to another implanting of virus in the importing countries (Boucher e Nouaille, 1996). Major histopathological lesions can be observed on liver (Abrantes et al., 2012) and it contains the highest viral concentration. There is primary liver necrosis and a massive disseminated intravascular coagulopathy in all organs and tissues (OIE, 2010). Haemorrhagic congestion can be seen in several organs, particularly in lungs, heart and kidneys. However trachea, spleen, digestive tract, chest and abdominal cavity, muscles and central nervous system are also affected (Abrantes et al., 2012).

All known strains of RHDV belong to one serotype, and this virus does not reproduce in cell culture. Small, non-enveloped viral particles are resistant to ether and chloroform (Fitzner et al., 2011).

The RHDV does not affect with the same intensity in all age groups, in adults rabbits mortality is around 90%, and 60% in rabbits whit ages between 4 and 6 months (Carvalho et al., 1993). The maternal antibodies have been suggested as one possible explanation for the survival in young rabbits (Abrantes et al., 2012). The incubation period of the disease in rabbits is three days (Carvalho and Almeida, 1990), and death usually occurs 12-36 hours after the onset of fever (OIE, 2010).

The origin and evolution of RHDV are not well understood. Although first reported in China in Angora rabbits imported from Germany, is was not clear if rabbits were already infected whit RHDV when they arrived in China, since the disease might have been previously observed in Germany, or if they became infected later in China. The idea of RHDV being of Chinese origin has been challenged by several studies. Indeed, these studies have shown that the pathogenic form of RHDV originated before 1984 and the Chinese strain isolated in 1984 had its origin in European isolates (Abrantes et al., 2012). In Europe, an outbreak (named “Mallatia X”) was reported in 1986 in Italy (Fitzner et al., 2011). Cases of RHD in the wild were reported in Spain in June of 1988. Soon afterwards the disease was reported in France, in Scandinavia by January 1990 and in Great Britain by August 1998, but had also been reported among domestic rabbits in the Russian Federation, in Middle East and parts of Africa, and in Cuba, Mexico and India (Cooke, 2002).

In Australia and New Zealand, where the rabbits are considered an important agricultural pest, as well as major threat to the endemic wild flora and fauna, rabbit haemorrhagic disease (RHDV) was soon considered as an agent for rabbit control (Abrantes et al., 2012). In Portugal, the disease was for the first time described in 1987 in Madeira Island. In the following years, the disease was reported in different islands of the Azores. In 1988, it was reported in Faial Island (Duarte et al., 2014) followed by a report in São Jorge Island in January of 1989 where this disease caused a mortality above 80%. In 1990, was reported the RHD in Santa Maria Island (Carvalho and Almeida, 1990) and in the same year RHD wiped out rabbit population in most of Azores Archipelago islands (Duarte et al., 2014).

In Portugal mainland, the first cases date back to 1989 (Duarte et al., 2014). Recently, rabbit Azorean samples have been used for genotyping studies, in order to integrate de RHDV in the existing genogroups. All the tested strains of RHDV obtained from Azores were identified as belonging to G5 genogroup, these G5 strains, seem to be the dominant group in these Atlantic islands (Duarte et al., 2014).

Hardly any commercial assays are yet available for detection many important pathogenic agents. Consequently, it is often necessary to develop in-house assays, which need adequate optimization and validation before they are introduced in routine diagnostics. The fluorescent-based quantitative real time polymerase chain reaction (RT-qPCR) is the technique that combines the advantage of conventional PCR with a real-time detection of the amplification products. Such RT-qPCR assays have been adapted and developed for various types of studies. Assay validation criteria are the characterizing traits of an assay that represent decisive factors, measures or standards upon which a judgment or decision may be based. Validation is a process that determines the fitness of an assay, which has been properly developed, optimized and standardized, for an intended purpose (OIE, 2013). In order to obtain specific results to detect RHDV by reverse transcription (RT) qPCR, the availability of an accurate and reliable detection method is essential to limit the risk of false positive and false negative results.

The aim of this study was to optimize the identification of the RHDV by RT-qPCR in order to use this technique in our laboratory routine.

**Material and Methods**

**Samples**

The rabbit liver samples (n=8) used for this study were admitted to our laboratory for routine analysis, and their diagnosis was confirmed by national reference laboratory in Lisbon, National Institute of Agrarian and Veterinarian Research (INIAV). We also
used RHDV positive liver samples (n=2) from Office International des Epizooties (OIE) reference laboratory.

Firstly the dead rabbits were submitted to necropsy and samples of lung, liver, kidney, trachea and spleen were collected for histopathology and virology exam. Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Sections of 3 µm thick were stained with haematoxylin and eosin (H&E) for routine microscopical examination. The rabbit liver samples admitted to routine virology analysis were from domestic (n=2) and wild (n=6) rabbits originated from Terceira (n=6), Pico (n=1) and Graciosa (n=1) islands.

Nucleic acid extraction

A total of 0.02-0.04 g of liver tissue was homogenized with 1 ml of ribonucleases (RNases) free water, and centrifuged at 3000 rpm for 2 minutes. The volume of 100 µl from supernatant was used in the extraction of RNA with “Mag Vet Universal Isolation Kit” from Laboratoire Service International (LSI), according to manufacturer’s instruction. The extraction was performed automatically on Thermo Scientific KingFisher ML equipment.

Extracted undiluted RNA, and serial dilutions were stored at -20°C and used for the RT-qPCR.

Real-time reverse transcription

To realize the RT-qPCR, we used a probe (6986-7010) FAM-5’- CCAARAGCACRCTCCTGT GTTCAACCT-3’-TAMRA and a pair of oligonucleotide primers P7vp60 (6941-6961) 5’-ACYTGACTGAACTYATTGACG-3’ and P8vp60 (7044-7022) 5’-TCAGACATAAGAAAGCCATTGG-3’ as describe by Fitzner et al., 2011, Gall et al., 2007, OIE Terrestrial Manual, 2010 (GenBank Accession Number DQ205345). The assay was performed in 7500 Real-Time PCR System (Applied Biosystems) with one-Step reaction using Verso 1-Step qRT-PCR Low Rox kit (Thermo Scientific). The final mixture has the volume of 25 µl, containing: 0.25 µl of Verso Enzyme Mix, 12.5 µl of 2x 1-Step qPCR Low Rox Mix, 1.25 µl of RT Enhancer, 1.25 of Eucaryotic 18S rRNA endogenous control (VIC/TAMRA Probe, Primer Limited 20X TaqMan Life Technology), 2.5 µl of extracted RNA and 7.25 µl of DNases and RNases free water. The cycling were the same used on RT-qPCR, already described on this paper in 2.3.

Positive and negative control

Analysis of a negative control, simultaneously with the specimen, enables detection of possible contamination during the extraction or the amplification (Raymaekers et al., 2009). The positive controls are analyzed to verify that the method is capable of adequately recover and amplify the target (Cunha and Inácio, 2014). In this study were used a RHDV RNA-positive control, and a RHDV RNA-negative control. The RHDV RNA-positive control corresponding to a reference liver sample sent from OIE laboratory and the RHDV RNA-negative control corresponding to a liver sample admitted to our laboratory for routine analysis and confirmed as RHDV RNA-negative in INIAV. The RHDV RNA-positive and RHDV RNA-negative controls were extracted the same way that liver samples as described in 2.2.

Equipment

All the equipment used in the validation process were properly maintained and calibrated. To amplify the extracted RNA on RT-qPCR were used clear plates sealed with “Optically Clear Adhesive Seal Sheets” (Thermo Scientific). All reagents used are in within the expiring date.

Validation

Repeatability and reproducibility of RT-qPCR assays

The repeatability (intra-assay precision) is the closeness of agreement between successive independent results, obtained under identical con-
ditions and gives important information about the assay before further validation tests (OIE, 2013). It refers to the precision and robustness of the assay with the same samples repeatedly analyzed in the same assay (Bustin et al., 2009). Repeatability was assessed by the same operator during the same work session, using three individual samples. Five individual aliquots obtained from starting samples were extracted and amplified as an independent sample. The intra-assay variability was calculated by assessing the homogeneity among replicates by the percentage of total variance obtained with replicas of different samples. The repeatability was expressed as SD (standard deviation) and %CV (percent coefficient of variation) for the C<sub>t</sub> variance obtained to each sample.

Reproducibility (inter-assay precision) is the closeness of agreement between successive and independent results (OIE, 2013), obtained under variations of measurement conditions. It refers to the variation in results between runs or between different laboratories (Bustin et al., 2009). Reproducibility was assessed by two operators during two different work sessions, using three individual samples. Three individual aliquots obtained from the starting samples were extracted and amplified as an independent sample. The reproducibility was expressed as the SD and %CV for the C<sub>t</sub> variance obtained to each sample.

Specificity

To prove the specificity, that is the capacity of the assay to detect an analysis or genomic sequence that is unique to a targeted organism, and excludes all other known organisms that are potentially cross-reactive (OIE, 2013), were tested a panel of pathogens. The pathogens were *Neospora Caninum*, Bovine Viral Diarrhea Virus (BVDV), *Mycoplasma bovis*, Bovine Herpes Virus 1 (BHV 1), *Chlamydophila* spp., classical RHDV, RHDVa, RHDV2 and RHDV RNA-negative liver samples.

Limit of detection and PCR efficiency

To determine the limit of detection (LOD) was used a reference RHDV RNA-positive liver sample from OIE laboratory. This positive liver sample was extracted and the product of the extraction was diluted in a RHDV RNA-negative liver sample according to a range of 10, until signal extinction. The undiluted sample and the serial dilutions were amplified in triplicate. The results were plotted as a standard curve constructed by plotting the log of the dilution factor against the C<sub>t</sub> value obtained during the amplification of each dilution. The equation of the linear regression line, along with Pearson’s correlation coefficient (r) and the coefficient of determination (R<sup>2</sup>) was used to evaluated how well the experimental data fit standard curve and how linear the data are.

The PCR efficiency is dependent on the assay, the master mix performance, and sample quality. Generally, efficiency between 90 and 110% is considered acceptable which corresponds to a slope of between -3.58 and -3.10 (Cunha and Inácio, 2014).

The efficiency (% Efficiency) was estimated through a linear regression of the dilution curve. According the formula: %Efficiency = \((10^{(-1/slope)} - 1) \times 100\), and the amplification efficiency (E) according the formula: \(E = 10^{(-1/slope)}\).

Results

Repeatability and reproducibility

Mean C<sub>t</sub> values, SD and %CV obtained with RT-qPCR carried out on total RNA extracted from RHDV RNA-positive samples and RHDV RNA-negative samples are shown in Table 1. Very low variation was obtained in the intra-assays to detect RHDV RNA (0.6291-0.6595%), and to detect 18S rRNA (0.4591-0.7962%) indicating that the method is highly repeatable. The 18S rRNA was detected in all samples what ensure adequate efficiency of RNA extraction and confirms the absence of PCR-inhibitors in each sample.

<table>
<thead>
<tr>
<th>Repeatability</th>
<th>Samples tested to RHDV</th>
<th>Samples tested to 18S rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Sample 1</td>
<td>5</td>
<td>16.57</td>
</tr>
<tr>
<td>Sample 2</td>
<td>5</td>
<td>17.40</td>
</tr>
<tr>
<td>Sample 3</td>
<td>5</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reproducibility</th>
<th>Samples tested to RHDV</th>
<th>Samples tested to 18S rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Sample 1</td>
<td>3</td>
<td>15.21</td>
</tr>
<tr>
<td>Sample 2</td>
<td>3</td>
<td>15.91</td>
</tr>
<tr>
<td>Sample 3</td>
<td>3</td>
<td>-</td>
</tr>
</tbody>
</table>

Sample 1: RHDV RNA positive; Sample 2: RHDV RNA positive; Sample 3: RHDV RNA negative. N= number of replicates.
and validate the negative results. Furthermore the high reproducibility of RT-qPCR determinations is reflected in the standard deviation values obtained.

**Limit of detection**

The lowest limit of detection for series of RNA dilution of RHDV RNA-positive sample was found in $10^{-5}$ dilution (Fig. 1), to $C_T=31.67$. A mean $C_T$ values from 14.34 at undiluted sample to 31.67 at $10^{-5}$ dilution were obtained. At the highest dilution ($10^{-4}$), $C_T$ values fluctuated around 31.47 and 31.67 (Table 2). The optimal dilution of cDNA ($C_T=18-25$) was obtained with first 3 dilutions. First five sets of replicates are good, but then variability is high when cDNA is too diluted.

The 18S RNA was detect in all samples. A mean $C_T$ values from 26.29 at undiluted sample to 13.43 to diluted samples were obtained (Table 3; Fig. 2).

**PCR efficiency**

At the end of the each cycle, the amplicon copy number increased 1.954-fold ($E=1.954$), or 95.4% of the template was amplified, indicating that 95.4% of the product was doubled with each cycle. The $R^2 = 0.998$ reflects a good linearity of the standard curve (Fig. 3).

**Specificity**

The specificity was confirmed in vitro since no amplification was achieved with the pathogens *Neospora Caninun*, *BVDV*, *Mycoplasma bovis*, *BHV 1*, *Chlamydophila* spp, RHDV 2 and with RHDV negative samples (Table 4). The $C_T$ values to RHDV RNA-positive samples ranged from 15.42 to 30.18, with a mean value 22.62 (SD=6.56). The 18S RNA was detect in all samples, a $C_T$ mean value 21.89 (SD=5.12) was obtained to RHDV RNA-positive samples and a $C_T$ mean value 15.47 (SD=0.25) was obtained to RHDV RNA-negative samples.
**Table 4 - Specificity of the RT-qPCR**

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>N</th>
<th>Positive fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neospora Caninum</td>
<td>1</td>
<td>No</td>
</tr>
<tr>
<td>BVDV</td>
<td>1</td>
<td>No</td>
</tr>
<tr>
<td>Mycoplasma bovis</td>
<td>1</td>
<td>No</td>
</tr>
<tr>
<td>BHV 1</td>
<td>1</td>
<td>No</td>
</tr>
<tr>
<td>Chlamydia spp</td>
<td>1</td>
<td>No</td>
</tr>
<tr>
<td>RHDV (classical)</td>
<td>3</td>
<td>Yes</td>
</tr>
<tr>
<td>RHDVα</td>
<td>1</td>
<td>Yes</td>
</tr>
<tr>
<td>RHDV 2</td>
<td>1</td>
<td>No</td>
</tr>
<tr>
<td>RHDV negative samples</td>
<td>5</td>
<td>No</td>
</tr>
</tbody>
</table>

N: Number of samples

**Necropsy and histopathological examination**

According necropsy and histopathology examination lungs presented moderate to severe congestion, petechial or larger haemorrhages irregularly distributed and affecting partial or complete lobes. The nostrils presented severe congestion and hemorrhages. The thymus, kidneys and spleen showed also congestion. Liver were slightly enlarged, soft and pale. The histopathology of the lungs revealed diffused congestion, alveolar oedema and moderate to severe intralveolar haemorrhage (Figure 4). Histopathology of the liver revealed acute and widespread necrosis with dissociation of the hepatic cords (Figure 5).

**Discussion**

In recent years, various qPCR methods have been implemented and applied to the diagnosis of human and animal disease. The RT-qPCR methods offer certain advantages over conventional RT-PCR. It avoids the use of agarose gel electrophoresis, therefore decreasing the risk of contamination, and is suitable for large scale testing and automation.

For implementation of a test procedure for routine use, it is essential to set up validation methodology. The aim of this study was to optimize the detection of RHD virus by RT-qPCR in order to use this technique in the routine of laboratory.

The RNA extraction method used is well adapted to tissue samples as we can confirm by the use of 18S rRNA. Analysis of the negative control, simultaneously with the samples, demonstrated no contamination during the extraction and amplification. Analysis of positive controls demonstrated that the master mix and reagents were prepared correctly in order to produce amplification of the target nucleic acid.

It was shown that the lowest limit of detection of viral RNA extracted from the liver of rabbit infected was found in $10^{-5}$ dilution, with $C_t$ mean value $C_t=31.67$. The optimal dilution of cDNA was established between $C_t$ 18-25. The hallmarks for an optimized RT-qPCR assay were reached in this study, the reaction efficiency found was 94.5%, the $R^2$ value was 0.998 and consistency across samples (reproducibility) and replicates reactions (repeatability) was obtained.

In conclusion, this assay proved to be very effective, detecting classical RHDV and RHDVα in biological materials and not RHDV 2. The application of the studied method of optimization RT-qPCR to RHDV detection can facilitate replication of experiment by others veterinary laboratories. Furthermore the results of this study indicate that RT-qPCR method could be used as a rapid method for detection RHDV in liver samples. The next step is develop an assay to detect RHDV 2 by RT-qPCR in LRV of Azores.

After the optimization, the internal quality control like the amplification of an IC, analysis of a negative control, a statistical follow-up of a positive control (reference material) is essential implement in routine of a laboratory as well as the external quality assessment like external proficiency programs, test blind samples, or exchange of samples with other laboratories.

**Acknowledgments**

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