A RT-PCR BASED STRATEGY TO IDENTIFY RABBIT HAEMORRAGIC DISEASE VIRUS

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ABSTRACT

Rabbit Hemorrhagic Disease Virus (RHDV) and European Brown Hare Syndrome Virus (EBHSV) are two virus belonging to the *Lagovirus* genus. They cause a hemorrhagic disease in rabbits and hares, respectively. The high mortality (up to 100%), and the great contagious potential of the viruses make them very dangerous for both wild and domestic animals. The viruses have a number of variants, and recently a new RHDV variant has been widely circulating among rabbits, mainly in France. The variability of viruses may cause some problems in the molecular diagnosis. We set up a RT-PCR based method which could widen the efficacy of the existing molecular techniques, as it could detect simultaneously the variants of RHDV, including the French variants, and the EBHSV, furthermore discriminating the two viral species.

Keywords: Rabbit hemorrhagic disease virus, european brown hare syndrome virus, detection, RT-PCR

INTRODUCTION

Rabbit Hemorrhagic Disease (RHD) is an infectious disease which is highly contagious for domestic and wild rabbits, with a great impact on both economy and ecology. The typical pathological lesion is the degenerative and hemorrhagic necrosis of the liver, which is usually observed after 16-48 hours since the infection (Hu *et al.*, 2010). Death usually occurs between 2 and 3 days post-infection, although some rabbits may live for several days before they die. Morbidity and mortality rates are very high, as 90% to 100% of the infected animals die (Belz, 2004). A similar clinical picture characterizes the European Brown Hare Syndrome (EBHS), but it only affects hares and exhibits lower mortality rates (Bergin *et al*., 2009; Bascunana *et al*., 1997).

Rabbit Hemorrhagic Disease and EBHS are caused by two viral species belonging to the *Lagovirus* genus, *Caliciviridae* family: the Rabbit Hemorrhagic Disease Virus (RHDV) and the European Brown Hare Syndrome Virus (EBHSV), respectively (OIE, 2008).

Those are non-enveloped viruses whose positive-strand RNA genomes are 7.5 to 8.3 kb in size. The genomic organization and the viral proteins of EBHS and RHDV are also similar in many aspects. The genome architecture of both RHDV and EBHSV distinguishes them from the other members of the family *Caliciviridae*, as they harbor two ORFs encoding a polyprotein and a minor structural protein. The former is finally hydrolyzed into 8 products including 7 nonstructural proteins and 1 structural protein named as VP60 (Cappucci *et al.,* 1996; Tian *et al.,* 2011).

Despite their similarities, the two viruses are well distinct, insomuch that cross-infection does not occur and vaccines do not cross-protect rabbits and hares (Cappucci *et al*, 1996).
Both viruses have a number of variants (Strive *et al*., 2009). Recently, a new variant of RHDV (hereafter, French variant) has been reported in France, where it has been widely circulating (Le Gall-Reculé *et al*., 2011a).

A prompt laboratory diagnosis is very useful, and the enzyme-linked immuno sorbent assay (ELISA) is among the commonly used methods (Bascunana *et al*., 1997). Recently, molecular based strategies for detection and identification of RHDV and EBHSV have been developed, but in some cases they fail in detecting the new variants.

In this paper we describe a RT-PCR strategy for the simultaneous detection of either RHDV, including the French variant, or EBHSV.

MATERIALS AND METHODS

Tissue samples and RNA extraction

Samples of liver from naturally RHDV infected rabbits and naturally EBHSV infected European brown hares were kindly provided by the Istituto Zooprofilattico Sperimentale delle Venezie, diagnostic section of Treviso, Italy. The viruses had been previously identified and characterized and the variant had been assessed. After an initial screening, we used three samples of rabbits naturally infected by the French variant, three samples infected by different RHDV variants (hereafter, classical variants) and three samples infected by EHBSV. Total RNA was extracted from the selected samples by using the RNeasy Mini Kit (Qiagen, Milan, Italy), according to the manufacturer's instructions.

Genome comparisons and primer design

The nucleotide sequences of the reference genomes of RHDV (RefSeq NC_001543) and EBHSV (RefSeq NC_002615) from GenBank were compared by Artemis Comparison Tool (Wellcome Trust Sanger Institute, Hinxton, UK) in order to delimitate the variable and conserved regions. A minimum cut off score of 70 was imposed.
The amino acid sequences of the protein VP60 and the corresponding nucleotide sequences were aligned by the Clustal W algorithm implemented in MEGA 5.0. Primers were designed using the Oligo 6.4 software (Molecular Biology Insights, Cascade, Colorado, USA). Sequences of primers and their locations on the RHDV reference genome RefSeq NC_001543 are listed in Table 1.

Table 1. Primers used in this study

Primer	Sequence (5'-3')	Primer location[a]
RHDVf	TGT AYG CTG GMT GGG CTG GTG G	5630-5651
RHDVr	TGG GRC GYA RRT CYG GCA TGG TG	5808-5830

[a] Primer locations are referred to RHDV reference genome RefSeq NC_001543

RT-PCR

One μL of total RNA extract was used as template in the subsequent RT-PCRs. The reactions were carried out by using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Milan, Italy) according to the manufacturer's instructions. Primers were used at a final concentration of 0.2 μM.

The reactions were performed in a Mastercycler (Eppendorf AG, Hamburg, Germany) as follow: the synthesis of cDNA, conducted at 48.5 °C for 30 min was followed by the inactivation of RT enzyme at 94 °C for 2 min and by 40 cycles of denaturation (15 s) at 94 °C, annealing (30 s) at 54.5 °C and extension (1 min) at 68 °C. Each assay was carried out in duplicate and a negative control without RNA was added. PCR results were analyzed by electrophoresis at 7.5 V/cm for 60 min in 1.5% agarose gel followed by staining with ethidium bromide 0.5 μg/ml. Stained agarose gels were exposed to UV and image were digitalized by mean of a GelDoc-It Imaging System (UVP, Upland, CA, US).

Specificity test

The pair of primers was analyzed for its specificity *in silico* by using the NCBI Primer-BLAST, available online at the address http://www.ncbi.nlm.nih.gov/tools/primer-blast, paying attention to those RNA viruses which usually infect rabbits or hares. Therefore we checked the specificity of the primers, among

others, against the *Leporipoxvirus* genus, the *Rotavirus* genus, the *Astroviridae* family. The specificity was determined in conditions of high and low stringency, obtained by properly adjusting the specific parameters, namely, at least 2 total mismatches to unintended target, including 2 mismatches within the last 5 bps at the 3′ end, ignoring targets which have 7 or more mismatches to the primer (high stringency); and at least 4 total mismatches to unintended target, including 4 mismatches within the last 4 bps at the 3′ end, ignoring targets which have 9 or more mismatches to the primer (low stringency).

Cloning and sequencing

The products of the RT-PCR were cloned in pGEM-T Easy cloning vector (Promega, Milan, Italy), according to the manufacturer's protocol. The cloned products were sequenced using the Big Dye Terminator v3.1 and sequence determination was performed on an Applied Biosystem ABI 3100 at the facilities of Bmr Genomics (Padova, Italy). The identification of the products was carried out by comparison with the sequence in GenBank, after the removal of primer sequences.

RESULTS AND DISCUSSION

In order to find a suitable region for the primer design, comparisons were performed among the RHDV genomes and between RHDV and EBHSV genomes submitted in GenBank. We found a variable region flanked by two conserved regions in the locus encoding for the VP60 protein. By analyzing the VP60 amino acid sequences from GenBank, we selected the most conserved regions. In particular, one of them was included between the positions 5617 and 5685 of the RHDV genome published as RefSeq with accession number NC_001543, and another laid between 5806 and 5832. Based on the nucleotide sequences of those regions we designed a pair of primers introducing a few degenerations to allow the amplification even in presence of some reported point mutations.

The RT-PCR protocol was tested with three samples for each of the RHDV classical variant, the RHDV French variant and the EBHSV. The RT-PCR returned a 201 bp amplicon from each classical and French variants of RHDV, and a 552 bp amplicon from the EBHSV sample (Figure. 1).

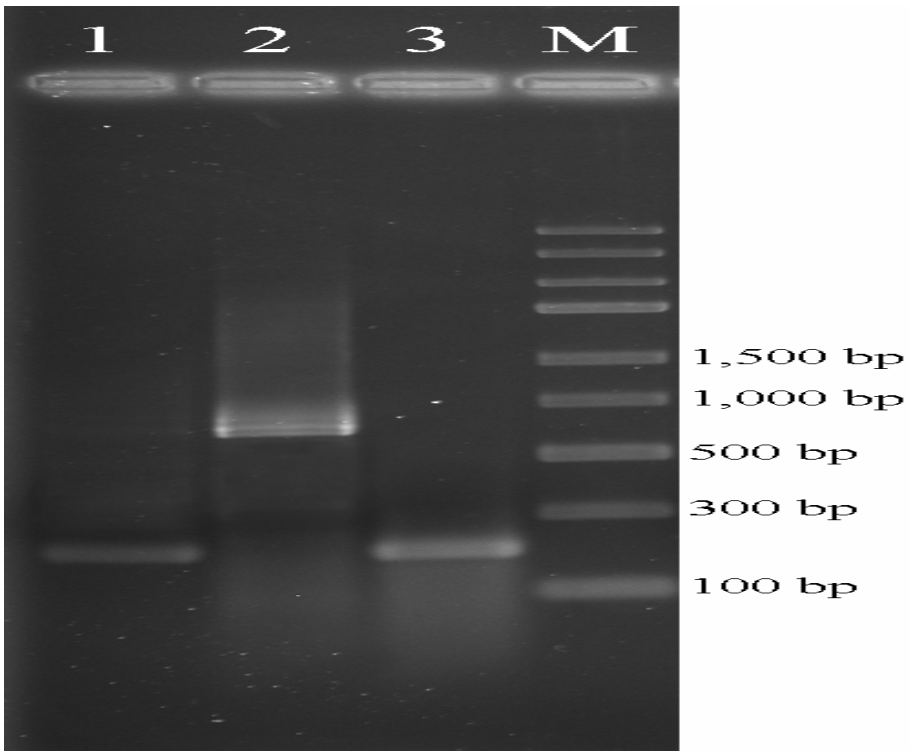

Figure 1. RT-PCR products achieved by using the RHDVf/RHDVr primers described in this study. 1). RHDV classical variant; 2) EBHSV; 3) RHDV French variant; M) Marker GeneRuler Express DNA ladder (Fermentas, Milan, Italy)

The RT-PCR products from classical and French variants of RHDV, although equal in length, share only the 85% of their nucleotide sequence. The BLAST analysis of such sequences confirmed that they matched the expected regions. In particular, the amplicon from the classical variant best matched (98% identity) with the RHDV strain Triptis (GenBank accession number EF558583), while the amplicon from the French variant best matched with the RHDV strain Rossi (GenBank accession number EF558584) with a lesser similarity (88% identity). We could not compare it with sequences from the French variants because, to our knowledge, there are no nucleotide sequences of such strains in GenBank. The amplicon from EBHSV best matched (94% identity) with the corresponding fragment of the EBHSV strain 0603 VP60 gene (GenBank accession number AM933648).

The exclusivity test was conducted *in silico* by Primer-BLAST, and there were no significant matches of the primers with the genomes of the viral families and genera included in the search.

In the light of those results, the RT-PCR we designed appears to be a useful alternative to the molecular protocols which are commonly used for detection and identification of RHDV and EBHSV. Among them, the real-time RT-PCR strategy proposed by Gall *et al*. (2007) correctly identifies both the RHDV and RHDVa subtypes of the classical variants, but some users reported that its efficacy is somehow impaired with the French variants. The RT-PCR protocol described by Le Gall-Reculé *et al*. (2011b) circumvents this problem, as it is effective against a wider panel of variants, including the non-pathogenic and the French variants. However, it does not appear suitable for the detection of EBHSV. Indeed, the latter may be detected by specific RT-PCR protocols, such as those proposed by Bascunana *et al*. (1997) and Le Gall-Reculé *et al*. (2001).

The protocol described in this paper seems to reach a good balance between sensitivity and specificity. In fact, the RT-PCR with the designed primers returned a positive result from the classical and the French variants of RHDV as well as from the EBHSV. Furthermore, the *in silico* analysis support the absence of aspecific annealing.

In addition, the protocol allows us to discriminate between RHDV and EBHSV, since the size of amplicons from the two viruses are different, being 201 bp and 552 bp, respectively.

This may be useful in simplifying the laboratory procedures for the detection of viruses, as a single protocol and pair of primers may reveal the two species in the biological samples. Furthermore, since the amplicon size and the melting temperatures of the primers are compatible with real time RT-PCR systems, the protocol may be easily adapted to perform quantitative PCR or to set up a discriminative technique based on the melting temperatures of the amplicons, in order to distinguish the classical and French variants. We are currently aiming our efforts to set up and validate this strategy.

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