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DEVELOPMENT OF AN IMMUNOCAPTURE-RT-PCR ASSAY FOR THE DETECTION AND MOLECULAR EPIDEMIOLOGY STUDY OF RABBIT HAEMORRHAGIC DISEASE VIRUSES (RHDV)

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ABSTRACT

Rabbit Haemorrhagic Disease Virus (RHDV), a calicivirus, is the causative agent of a highly contagious and fatal disease of rabbits. We developed a new assay for the detection of RHDV based on viral purification by immunocapture and genomic amplification by reverse transcription-polymerase chain reaction (IC-RT-PCR). The assay is realized directly from the rabbit liver exudate obtained after thawing. This method combines the advantages of ELISA tests (rapidity) since immunocapture and RT reaction were carried out in a microtiter plate, and the advantages of assays based on the PCR reaction (sensitivity and sequencing). IC-RT-PCR assay was validated by comparison with our sandwich-ELISA used for rhdv diagnosis. By testing 10-fold serial dilutions of positive samples, we confirmed the higher sensitivity of the IC-RT-PCR. Moreover, 120 rabbit liver samples were analysed using the two methods and no divergent result was found. By simplifying sample preparation, IC-RT-PCR procedure is suitable for epidemiological studies conducted by nucleic sequence comparison. This work constitutes the first description of an IC-RT-PCR assay for the detection of a rabbit calicivirus.

INTRODUCTION

Rabbit haemorrhagic disease (RHD) is a highly contagious and fatal disease of both domestic and wild rabbits, with a high rate of mortality (60 to 90 per cent in adult animals). The disease, first described in China in 1984 (Liu et al., 1984) and subsequently in many other parts of the world, is responsible for great economic losses in rabbit production. The virus has been identified as a calicivirus (OHLINGER et al., 1990; MEYERS et al., 1991).

At present, laboratory diagnosis is based on enzyme-linked immunosorbent assays (ELISA) performed on rabbit liver (OHLINGER et al., 1990; Capucci et al., 1991). We have developed a sandwich-ELISA to detect RHDV, first in infected liver suspension (Guittre et al., 1995), then directly from the liver exudate obtained after thawing, in order to simplify the sample preparation step.

Nevertheless, to ensure the protective activity of vaccines and to develop appropriate diagnosis tests, it was necessary to determine the sequence diversity that exists among populations of RHDV isolates. For this purpose, several sensitive RT-PCR assays were developed in order to carry out phylogenetic studies (Guittre et al., 1995; NOWOTNY et al., 1997; Le Gall et al., 1998). However, the RT-PCR procedures are time-consuming and do not allow the processing of large numbers of samples.

Immunocapture-RT-PCR (IC-RT-PCR) is a procedure based on the PCR amplification of cDNA synthesized from the RNA of antibody-captured viruses. This test was first reported for hepatitis A virus detection (JANSEN et al., 1990) and successfully used for other RNA virus families (WETZEL et al. 1992, NOLASCO et al., 1993; CHEVALIER et al., 1995). The specificity of an IC-RT-PCR resides in both the interactions virus-antibody and primer-

template. Moreover, this approach simplifies sample preparation, involves fewer manipulations than conventional RT-PCR and is suitable to carry out molecular epidemiology studies.

In this study, we describe an IC-RT-PCR protocol for RHDV diagnosis that can also be used for RHDV phylogenetic studies, after sequencing of the PCR products.

MATERIAL AND METHODS

Virus samples

Liver specimens for isolation of RHDV were obtained from dead domestic and wild rabbits of various regions of France. Livers were stored at -20°C and were thawed at least once to obtain an exudate subsequently used in sandwich-ELISA and IC-RT-PCR assays.

Antisera

Positive chicken hyperimmune anti-RHDV serum was produced by immunisation of 6-week-old SPF chicken with RHDV purified as described in LE GALL et al. (1992). Positive rabbit hyperimmune serum was produced by immunisation of 10-week-old susceptible rabbits with inactivated infected rabbit liver homogenates. Negative rabbit serum was collected from rabbits proved to be seronegative against RHDV.

Sandwich-ELISA

The sandwich-ELISA was performed according to GuittÉ et al. (1995) with some modifications. The blocking step after the coating with chicken anti-RHDV serum was suppressed and we used liver exudate diluted 1:4 in PBS containing 0.2 per cent Tween 20 instead of the supernatant obtained after centrifugation of homogenised liver.

Oligonucleotide primers

The p33 and p34 hybrid primers described in Le Gall et al. (1998) were used. The first half of the primer corresponded to the standard sequencing primers (M13 and reverse primers) and the second half to the RHDV specific sequence, according to the strategy for direct sequencing of PCR products developed by VENDE et al. (1995). The primers allowed the amplification of a 501 bp genomic region, located at the 5' end of the gene encoding the RHDV capsid protein (VP60).

IC-RT-PCR assay

Polystyrene microplates (96-well plates) were coated with 100 μl of chicken anti-RHD hyperimmune serum (diluted 1:1000 in carbonate buffer, pH 9.6) for one hour or overnight at 37°C . To avoid cross-contamination between wells, we only used one well out of 4. The wells were washed three times with PBS containing 0.2 per cent Tween 20 (PBS-Tween) then, 100 μl of exudate were added and incubated for one hour at 37°C . The wells were washed six times with PBS-Tween with care taken to avoid cross-contamination. The reverse transcription reaction was carried out in the plate by adding to each well 25 μl of the RT mixture containing 50 units of Murine Moloney leukemia virus reverse transcriptase (Gibco-BRL) and 25 pmol of oligo-dT as a primer. The plate was incubated 1 hour at 37°C . Then, the transcription solution was transferred from the microplate wells to 0.5-ml tubes and 4 μl of the cDNA solution were added for the PCR reaction. The remaining cDNA solution was stored at -20°C . Amplification was accomplished in microtubes under 20 μl containing 10 pmol of each PCR primer and 0.3 unit of Tub polymerase (Amersham). Five preliminary cycles of 30 s at 92°C , 30 s at 49°C and 1 min at 72°C were made, permitting the hybridation of the 3' half of the chimeric primer. This was followed by 25 cycles of 30 s at 92°C , 30 s at 65°C and 1 min at 72°C for the annealing of the whole primer. The reaction was completed with a final elongation step of 10 min at 72°C .

Detection of the amplified products

Amplified DNA products (predicted size of 581 bp) were analysed by conventional

electrophoresis techniques on agarose gel (Sambrook et al., 1989).

RESULTS AND DISCUSSION

IC-RT-PCR procedure

Because of the inability to replicate RHDV in cell cultures, viral particules were directly captured from infected liver exudate obtained after thawing.

The method described in this paper is shown in Figure 1. The major steps are: immunocapture of viral particles in the wells of a microtiter plate coated with specific antibodies, direct RT reaction, then transfert of the RT solution in a microtube and PCR. Agarose gel analysis of the amplification products revealed the presence of the predicted 581 bp band in positive samples.

As reported in the next section, all the samples that proved positive in sandwich-ELISA were also positive by IC-RT-PCR test. This suggests that the inhibitory substances of RT-PCR assay, possibly present in the exudate, were easily eliminated by immobilization of the virions on a solid support and the subsequent washing. This observation is in agreement with previous works (GRAFF et al., 1993; NOLASCO et al., 1993; CHEVALIER et al., 1995) and constitutes one of the advantages of the technique.

After having tested several different protocols, we finally performed the RT directly on the retained material without any previous thermal or chemical disruption of the virus particles, according to NOLASCO et al. (1993). Because removal of the virus capsid was not necessary for the exposure of the viral genome to the RT reaction, immobilization, washing and RT-PCR can be carried out sequentially in a single tube or in a well of a microtiter plate (NOLASCO et al., 1993). We chosed to develop an assay using microtiter plates to facilitate the washing steps and in the perspective of carrying out all steps of IC-RT-PCR in the same well.

We envisage performing a fluorometric detection of amplified products to develop a method that can be entirely automated for routine diagnosis. Because our thermocycler is not equipped to receive a microtiter plate, we had to transfer RT solutions in microtubes. Moreover, since only 4 μ l of cDNA solution was used for PCR, we had the possibility to perform PCR with other primers in order to amplify different parts of the genome (data not shown).

Comparison of the IC-RT-PCR and the sandwich-ELISA

Comparison of sensitivity of the ELISA and IC-RT-PCR was undertaken on parallel experiments by using 10-fold serial dilutions of positive samples, in order to determine the lower limit of detection of RHDV by each methods. In ELISA, the end-point dilutions were from 10^{-2} to 10^{-4} , whereas PCR products were detectable up 10^{-3} to 10^{-6} -fold dilutions (data not shown). Thus, IC-RT-PCR was shown to be 10 to 100-fold more sensitive than sandwich-ELISA.

One hundred and twenty rabbit liver samples found RHDV-positive or negative by using the sanwich-ELISA were tested by IC-RT-PCR. As shown in Table 1, there was a very good correlation between the results of the IC-RT-PCR and the sandwich-ELISA tests. No divergent results were found: 63 livers that were positive by the ELISA were also positive by PCR, and 57 samples were negative by the two methods. No false negative or positive PCR results were noticed.

Table 1: Comparison of Sandwich-ELISA and IC-RT-PCR for the detection of RHDV in rabbit samples.

		IC-RT-PCR		Total
		Number of positive samples	Number of negative samples	
Sandwich-ELISA	Number of positive samples	63	0	63
	Number of negative samples	0	57	57
	Total	63	57	120

Application of IC-RT-PCR to diagnosis and phylogenetic analysis

We confirmed that IC-RT-PCR constitutes a reliable tool for the diagnosis of RHDV and is more sensitive than sandwich-ELISA for the detection of low concentrations of virus. The technical simplicity of IC-RT-PCR makes it applicable to routine diagnosis and may be carried out in equipped laboratories from now. However, use of more expensive reagents and currently detection of the amplified products by gel electrophoresis, may constitute limiting factors of this method compared to ELISA. If necessary, further works could be undertaken to amplify the DNA and detect the PCR products in a same microplate, in order to reach an equivalent degree of automation as ELISA.

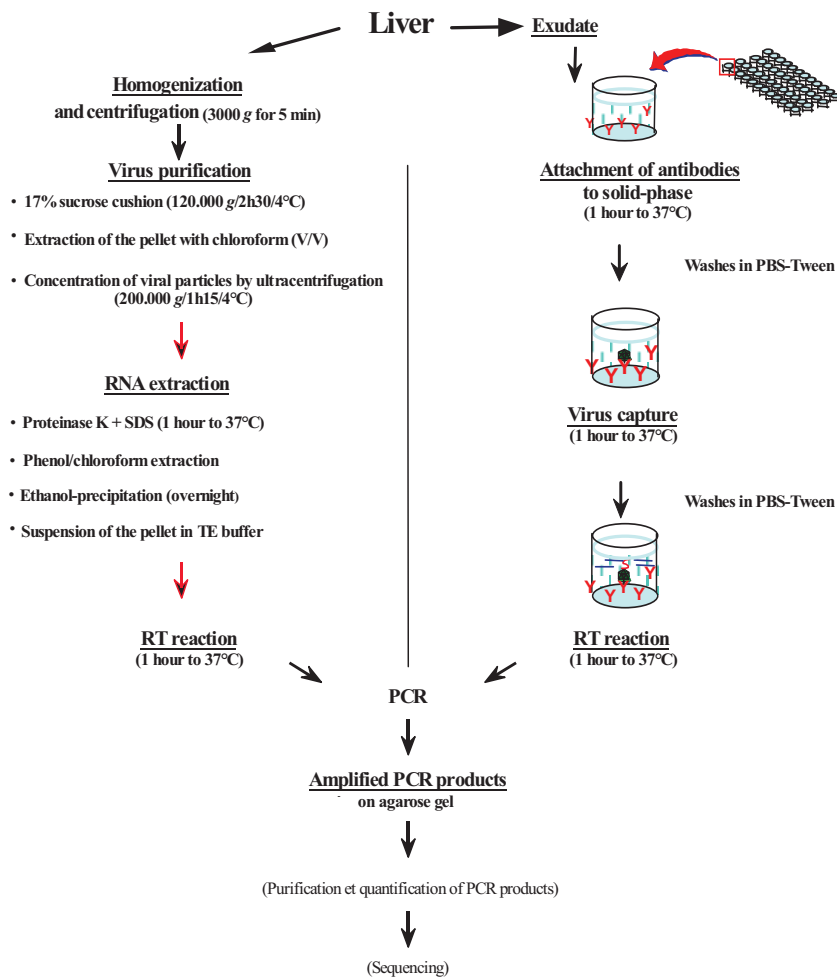
Until now, phylogenetic analysis were carried out from RHDV nucleic acid extracted and purified as described in VENDE et al. (1995) (Figure 1). Briefly, small liver fragments from an RHDV-infected rabbit were homogenized and centrifuged, then the supernatant was purified onto a sucrose cushion and the resulting pellet was extracted with chloroform. The viral particles were concentrated by ultracentrifugation and the pellet treated with proteinase K. The nucleic acids were extracted twice with phenol/chloroform and then ethanol precipitated.

For the RT-PCR, we used the sequencing strategy developed by VENDE et al. (1995) to choose the PCR primers. It was based on the use of hybrid primers including a fluorescent-labelled universal 5' end. This procedure allowed to have the advantages of sequencing method using a classical dye-primer kit on an automated sequencer. It allowed us to carry out a phylogenetic study utilizing sequences encoding the capsid protein of 56 virus isolates collected from various parts of France between 1988 and 1995 (Le Gall et al., 1998), but proved to be very time consuming.

The IC-RT-PCR procedure is not more time consuming than ELISA and can be used easily in molecular epidemiology studies. Moreover, we showed that it was more sensitive. Indeed, the IC-RT-PCR assay allowed us to carry the phylogenetic study by analysing 51 new RHDV isolates collected in France between 1995 and 1999 in only a few weeks.

Recently, we adapted this procedure to the detection of European Brown Hare Syndrome (EBHS) virus, a calicivirus responsible of a severe necrotic hepatitis in Hare and closely related to RHDV (CHASEY et al., 1992; LE GALL et al., 1996). A phylogenetic study of EBHSV is undertaken currently.

Figure 1: Scheme of the RHDV IC-RT-PCR assay (right) and comparison with the classical method of RHDV acid nucleic extraction (left).



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