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▶ **To cite this version:**

Patrice Vende, G. Le Gall, Denis Rasschaert. An alternative method for direct sequencing of PCR products, for epidemiological studies performed by nucleic sequence comparison. Application to rabbit haemorrhagic disease virus. Veterinary Research, 1995, 26 (3), pp.174-179. hal-02707706

HAL Id: hal-02707706
https://hal.inrae.fr/hal-02707706v1

Submitted on 1 Jun 2020

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Vet Res (1995) 26, 174-179
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Short note

An alternative method for direct sequencing of PCR products, for epidemiological studies performed by nucleic sequence comparison. Application to rabbit haemorrhagic disease virus

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(Received 5 August 1994; accepted 26 January 1995)

Summary — A sequencing strategy based on the use of commercially available fluorescent-labeled universal primers for directly sequencing polymerase chain reaction (PCR) amplified material has been developed. The PCR reactions were performed with hybrid primers, specific to the viral sequence and possessing the sequence of the standard sequencing primers at their 5' end (M13 and reverse primers). These amplified fragments were sequenced by a classical dye-primer kit on an automated sequencer. As opposed to the use of fluorescent dideoxynucleotides, this sequencing method yielded accurate, high-grade sequences and had several advantages. First, the intensity of the sequencing peaks was much more homogeneous with the dye primer method. In addition, the problem of altered electrophoretic mobility, which may occur during the sequencing of custom-synthesised fluorescent primers, was avoided. This method was successfully reproduced using several different sets of chimeric primers. We believe that it is suitable for epidemiological studies conducted by nucleic sequence comparison, as in the case of rabbit haemorrhagic disease virus, as well as in other systems.

automated sequencing / PCR / molecular epidemiology / rabbit haemorrhagic disease virus

Résumé — Méthode de séquençage direct de fragments amplifiés par PCR, adaptée aux études épidémiologiques réalisées par comparaison de séquences nucléiques. Application au virus de la maladie hémorragique du lapin. *Une stratégie de séquençage basée sur l'utilisation des amorces universelles de séquençage marquées par fluorescence et disponibles comme telles dans le commerce a été développée pour séquencer de l'ADN amplifié par* polymerase chain reaction *(PCR). Pour cela, les réactions de PCR ont été réalisées avec des amorces nucléotidiques hybrides, spécifiques de la séquence virale d'une part, mais possédant d'autre part à leur extrémité 5' la séquence des*

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amorces standard de séquençage (-21M13 et M13 reverse). Les fragments ainsi amplifiés ont été alors séquencés à l'aide d'un protocole classique utilisant les amorces fluorescentes universelles, sur un séquenceur automatique. Cette méthode de séquençage, qui s'oppose à l'emploi des didéoxynucléotides fluorescents, conduit à des séquences de bonne qualité et présente des avantages. Tout d'abord, l'intensité des pics des séquences est plus homogène avec la méthode des amorces universelles fluorescentes. Par ailleurs les problèmes d'altérations de mobilité électrophorétique survenant lors du séquençage effectué à partir d'amorces spécifiquement synthétisées et marquées pour l'expérience peuvent être évités par la méthode décrite ici. Cette technique a été reproduite avec succès en utilisant plusieurs jeux d'amorces chimériques différentes. Nous pensons qu'elle est adaptée aux études épidémiologiques menées par comparaison de séquences génomiques, dans le cas du virus de la maladie hémorragique du lapin, et qu'elle peut être étendue à d'autres systèmes.

séquençage automatique / amplification en chaîne par polymérase / épidémiologie moléculaire / virus de la maladie hémorragique du lapin

INTRODUCTION

The virus of the rabbit hemorrhagic disease causes high mortality in animals older than 2 months, in both wild and captive rabbit populations. (For a review see Ohlinger *et al*, 1993).

The whole genomes of 2 rabbit haemorrhagic disease virus (RHDV) isolates have been cloned and sequenced (Meyers *et al*, 1991; Rasschaert *et al*, 1995). The virus has a 7.5 kb genome consisting of a single-stranded, positive sense, polyadenylated RNA. The capsid is composed of a single protein, VP60, whose amino-acid sequence has been compared with those of other caliciviruses (Jiang *et al*, 1993). Little is known about the genetic variability of VP60 of RHDV. An epidemiological study that would determine the geographical and historical progression of the disease could contribute to the knowledge of RHDV VP60 gene variations. It could also lead to a preliminary evaluation of the efficiency of possible prospective vaccination campaigns performed with a single RHDV isolate, in the case where a good conservation of its capsid sequence exists.

To date, in the absence of cell culture system for RHDV, nothing is really known about the existence of several serotypes. For this reason, classical epidemiological approaches cannot be applied. To perform such a survey, it was thus necessary to sequence short regions of the capsid genome of several field RHDV isolates. For such an extensive undertaking, a direct, efficient and repetitive method was required.

For this reason, we have developed a fluorescent-labeled DNA sequencing strategy that uses commercially available M13 dye-primers. The polymerase chain reaction (PCR) steps were carried out with primers specific to the viral sequence, which possessed the sequence of the standard sequencing primers at their 5' end (–21M13 or ReverseM13). The PCR products were then simply sequenced with a dye primer kit on an automated sequencer.

MATERIALS AND METHODS

Small liver fragments from an RHDV-infected rabbit (CNEVA, unité de pathologie cunicole, Ploufragan, France) were homogenized and extracted twice with chloroform. The aqueous phase was layered onto a 17% sucrose cushion and centrifuged for 2 h at 100 000 *g*. The pellet was treated with proteinase K at 50 µg/ml in 20 mM Tris-HCl buffer pH 7.4, 1% SDS, for 30 min at 37°C. The nucleic acids were extracted twice with phenol/chloroform and were then ethanol-precipitated.

Genomic polyadenylated RNA was used to prepare the cDNA using Murine Moloney

leukemia virus reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA) with 50 pmol of oligo-dT as primer, according to the supplier's instructions.

The amplification mixture contained 10% of the cDNA obtained, 200 mM dNTP, 100 pmol of each PCR-primer, Tub-buffer and 5 units of Tub polymerase (Amersham).

The PCR-primers were 38 nucleotides (nt) long. The first 18 nt corresponded to the standard sequencing primers and the last 20 nt to the RHDV specific sequence (fig 1). Amplifications were performed on a Perkin Elmer/Cetus apparatus under the following conditions. First, 5 preliminary cycles were made (1 min at 92°C, 1 min at 50°C and 2 min at 72°C); the annealing temperature of 50°C permitted the hybridation of the specific 3' half (20 nt) of the chimeric primer. Secondly, 25 cycles were made (1 min at 92°C, 1 min at 65°C and 2 min at 72°C); these were adapted to the annealing of the whole primer (38 nt). Finally, the extension step (10 min at 72°C) was performed.

The PCR products were then cleansed of the PCR reaction medium before being sequenced using a Quiaquick PCR purification kit (Quiagen, chatsworth, CA, USA) according to the supplier's instructions. These spin columns permitted the removal of unincorporated nucleotides, excess of PCR primers, enzymes and all reagents.

Aliquots of 200 ng of this DNA were used as sequencing template with the Ampli Taq Dye-Primer Cycle Sequencing Kit from Applied Biosystems (ABI/division of Perkin Elmer, Foster City, CA, USA) using an ABI catalyst labstation model. The reaction products were then loaded and analysed on an Applied Bioystems 373 automated DNA sequencer.

RESULTS

The use of the protocol described above permitted full length sequences of PCR products to be determined from both strands. The quality of the output was optimal: homogeneous signals, few ambiguities and a low baseline. Our technique provided accurate sequences that did not have too many ambiguous 'N' bases (2–3% for 450 nucleotides). It was reproducible; to date we have repeated and performed these sequence determinations on more than 30 RHDV isolates using 3 distinct sets of PCR primers. In many cases, the sequence determination was limited by the resolving power and the length of the gel. An example of the quality of sequencing result obtained with this method is illustrated in figure 2.

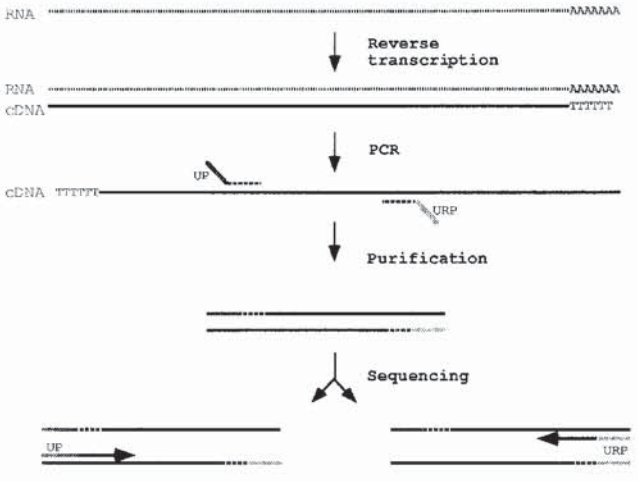

Fig 1. Strategy for amplifying and sequencing nucleic acid of RHDV isolates. The RNA was reverse-transcripted, the selected region was amplified by PCR, the resulting PCR-products were purified on a spin column, and the sequencing was carried out with Taq dye-labeled –21 M13 (UP) or reverse primer (URP) cycle sequencing kits.

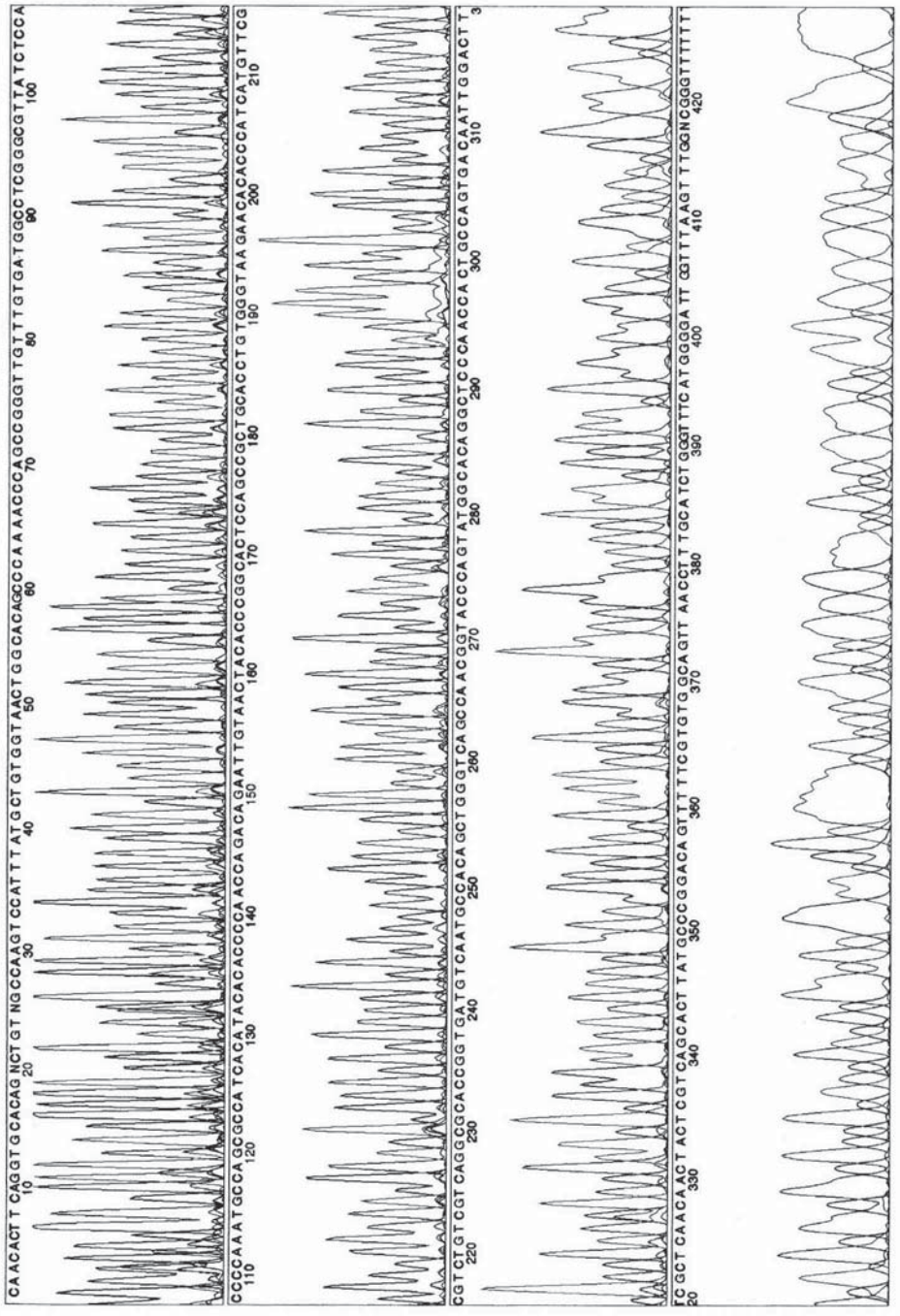

Fig 2. Example of sequencing plots obtained with this method. The sequence of the region 6480–6906 of an RHDV isolate was determined using a dye-labeled –21M13 primer.

DISCUSSION

A major problem encountered with the direct sequencing of double-stranded PCR products concerns the interference caused by PCR primers during the sequencing reaction. Some solutions have been proposed by other authors, such as: i) separation and purification of the PCR product by electrophoresis (Cao *et al*, 1993) or high-performance liquid chromatography (HPLC) (Chatterjee *et al*, 1993); ii) digestion of the PCR primers by mung bean nuclease before sequencing (Dowton and Austin, 1993); iii) asymmetric PCR (Murray, 1989); and iv) PCR with primers shorter than the sequencing primers, allowing discrimination to be based on the annealing temperature during the sequencing steps (Liu *et al*, 1993). However, the sequencing of PCR products purified by the above-mentioned methods with an automated sequencer requires the use of a dye-dideoxynucleotide sequencing protocol. At the present time, this Taq dye–terminator chemistry, which yields peaks of variable height, is not as efficient for sequencing more than 400 nucleotides, as the Taq dye–primer chemistry.

Furthermore, if some of the PCR primers remain present, they may compete with sequencing primers during the sequencing reaction. Moreover, the annealing temperature (50°C) used during the cycle sequencing is adapted to the melting temperature of M13 primers (18 nt) and not to those of the PCR primers (38 nt), which are then likely to hybridize in a non-specific manner. Since the PCR primers are unlabeled, the only detectable fragments during automated sequencing electrophoresis are those initiated by the sequencing dye-primers. The plots may therefore be slightly attenuated, while remaining perfectly readable, without having any multiple signals or background noise. Thus, with the dye–primer method, the absolute elimination of all the PCR primers is not critical for successful sequencing, as would be the case with the dye–terminator method.

The alternative option of labeling specific primers would need additional manipulations, because the 5'-terminal fluorescent labeling of primers other than the universal primers, leads to mobility shifts in the sequencing pattern, which have been reported as not being completely compensated by the ABI software. An empirical correction factor must therefore be determined for each set of new primers. This additional work can be avoided by using the method employed here.

This method is not only convenient for molecular epidemiological studies such as this one involving RHDV, but can also be easily applied to other biological models when serological methods are not available. Indeed, from a technical point of view, the apparent difficulty which occurs with the use of PCR primers, in which one part is constant (depending on M13 primers) and the other part is attached to the sequence that is going to be amplified, can be resolved by adapted PCR conditions; preliminary cycles during PCR facilitate the hybridation of the specific part. In addition, 3 series of amplifications were carried out in 3 distinct regions of RHDV genome, which led to the use of 3 different pairs of hybrid primers. All of these gave good results.

Concerning the epidemiological study of RHDV, 3 shorts regions (400–550 nt), located in the gene coding for the capsid (a part supposed to be variable) were chosen. Work has begun on the sequencing of these regions following this method, on more than 30 RHDV isolates from different locations in France and from different times (to be published). This will be completed with samples from other regions of the world. Finally, all of the data will be processed with sequence comparison software.

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