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* Manuscript

Characterization of the genome of human enteroviruses: design of generic primers for amplification and sequencing of different regions of the viral genome

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Abstract

Human enteroviruses are among the most common viruses infecting humans and can cause diverse clinical syndromes ranging from minor febrile illness to severe and potentially fatal diseases. Biodiversity and evolution of human enterovirus genomes are shaped by frequent recombination events. Therefore, identification and characterization of circulating strains of enteroviruses requires partial determination of different genomic regions.

This article reports the development of a simple method allowing amplification and partial sequencing of the P1, P2 and P3 genomic regions of field human enterovirus strains isolated in cell cultures, by performing PCR on cDNAs generated through a single RT reaction. A set of generic primers were designed and tested on a panel of 90 field and prototype viruses belonging to the five species of human enteroviruses. This assay was shown to amplify efficiently the targeted regions of all the 90 genomes tested. The generated amplicons were sequenced successfully without the need for gel purification.

This assay could be a precious tool for laboratories interested in molecular epidemiology and evolution studies implicating a great number of human enterovirus strains isolated from human or environmental samples.

Keywords: coxsackievirus, echovirus, genotyping, human enterovirus, poliovirus, random primer

1. Introduction

Human enteroviruses, members of the genus *Enterovirus*, family *Picornaviridae*, are among the most common viruses infecting humans. Although most infections with human enteroviruses are asymptomatic or result in mild infection, human enteroviruses can cause diverse clinical syndromes ranging from minor febrile illness to severe and potentially fatal diseases, such as acute hemorrhagic conjunctivitis, aseptic meningo-encephalitis, and acute flaccid paralysis (Palacios and Oberste, 2005).

Enterovirus virions are non-enveloped and contain a single positive-sense RNA genome of about 7,500 nucleotides (nt) with only one open-reading frame (ORF) flanked by untranslated regions (Figure 1A). This ORF encodes a single polyprotein. Co- and post-translational proteolytic processing gives rise to three precursors (P1-P3) secondarily cleaved in four structural proteins (VP1-VP4), which are assembled into the virion, and non-structural proteins, which are expressed in infected cells (Pallansch and Roos, 2001).

Historically, human enteroviruses have been classified into echoviruses (EV), coxsackievirus groups A and B (CV-A and CV-B), and polioviruses (PV), according to their phenotypic and pathogenic properties. Current taxonomy, based essentially on phylogenetic studies, divides human enteroviruses into 5 species (Hyypia et al., 1997): HEV-A (at least 17 serotypes), HEV-B (at least 56 serotypes), HEV-C (at least 13 serotypes), HEV-D (at least 3 serotypes) and the 3 serotypes of PV.

After isolation in cell culture, typing of human enteroviruses consists in identifying the serotype by seroneutralization. Because of their labour intensiveness, neutralization methods have been replaced by molecular tools in many laboratories. Indeed, a correlation between serotype and genotype for the P1 region of the genome has been demonstrated (Kottaridi et

al., 2005; Oberste et al., 1999). Therefore, several methods have been developed in order to determine partly the sequences encoding the VP1 protein (Bailly et al., 2000; Caro et al., 2001; Casas et al., 2001; Iturriza-Gomara et al., 2006; Nix et al., 2006; Norder et al., 2001; Oberste et al., 2003; Thoelen et al., 2003), which plays a key role in the constitution of the viral neutralization antigenic sites. Methods targeting other capsid-encoding regions, such as VP2 or VP4, have also been developed (Ishiko et al., 2002; Nasri et al., 2007). These methods were shown to be suitable for the purpose of determining the serotypes of field or clinical strains. However, the determination of the serotype appears insufficient for surveillance or research purpose, particularly for studying the molecular epidemiology and evolution of human enteroviruses. Indeed, recombination events between enteroviruses are known to be very frequent (Dahourou et al., 2002; Lindberg et al., 2003; Lukashev, 2005; Oberste et al., 2004; Oprisan et al., 2002; Santti et al., 1999), leading to a wide range of mosaic genomes inside human enterovirus species (Chen et al., 2007; Lukashev et al., 2003; Oberste et al., 2004; Rakoto-Andrianarivelo et al., 2007; Simmonds and Welch, 2006). Recombination is now considered as an important way of evolution of human enteroviruses (Chevaliez et al., 2004; Lukashev, 2005; Oberste et al., 2004), which can give rise to new viral genotypes exhibiting modified pathogenic properties. Thus, recombinant viruses associating capsid sequences of vaccine-derived strains of PV and sequences from HEV-C have been rendered responsible for several outbreaks of acute poliomyelitis (Anonymous, 2001a; Anonymous, 2001b; Kew et al., 2004; Rousset et al., 2003). Moreover, recombination could explain the failure of attempts to correlate serotypes and clinical symptoms in most human enterovirus-induced diseases.

While recombination is observed in the capsid-encoding region (Blomqvist et al., 2003; Bouslama et al., 2007; Martin et al., 2002), recombination events occur mainly in the P2 and P3 regions of the genome (Arita et al., 2005; Cuervo et al., 2001; King, 1988;

Simmonds and Welch, 2006). Therefore, typing approaches based solely on the capsid sequence appear to be insufficient for the survey of human enterovirus circulation and for the understanding of human enterovirus evolution, which require the determination of sequences of other genomic regions, particularly the non-structural protein-encoding sequences.

In order to make this determination easier, a simple method was developed, which allows amplification and partial sequencing of the genomic regions of each of the precursors P1 to P3 of field human enterovirus strains isolated in cell cultures, by performing PCR on cDNAs generated through a single RT reaction.

2. Materials and methods

2.1 Virus isolates

The prototype viruses were provided by the National Institute of Public Health and the Environment (RIVM) (Bilthoven, The Netherlands). The field isolates were from stools collected in Central African Republic and in Madagascar from 2001 to 2007. These isolates were typed by partial sequencing of their VP1-encoding gene following the method already described (Nix et al., 2006).

2.2 Sequence analysis

Human enteroviruses full-length polyprotein sequences retrieved from the GenBank database were used to design the primers. Viral amino-acidic conserved regions were identified by generating multiple alignment using CLC Combined Workbench 3.0 software (CLC bio, Aarhus, Denmark).

2.3 Virus preparation and RNA extraction

All work with infectious viruses was carried out in a BSL-2 facility. HEV-A and B were grown in RD cells, HEV-C and PV in HEp2c cells and HEV-D in GaBi cells (Kapsenberg, 1968).

Viral RNAs were extracted from 250 μL of culture supernatants using the High Pure Viral RNA kit (Roche Diagnostics, Meylan, France), following the manufacturer's instructions.

2.4 RT-PCR conditions

Primers (Eurogentec, Liège, Belgium) are listed in Table 1. Working solutions were prepared at a concentration of 20 μM, except for heptaN, which was dissolved at 100 μg.mL^{-1}.

cDNA synthesis was performed in a final volume of 10.5 μL using the SuperScript II reverse transcriptase (Invitrogen, Cergy-Pontoise, France); the reaction mixture contained 5 μL of purified viral RNA, 2 μL of 5X First-Strand Buffer, 0.01 M dithiothreitol (1 μL), 100 ng of the random primers heptaN (1 μL), 10 pmol of each dNTP (1 μL of a 10 mM mixture) and 100 U of enzyme (0.5 μL). The RT reaction mixture was then incubated at 25 °C for 10 min, 42 °C for 45 min and 95 °C for 5 min.

cDNAs were then used as templates for amplification in PCR carried out in a final volume of 50 μL that included 5 μL of 10X PCR Buffer, 200 μM of each dNTP, 50 pmol (2.5 μL) of each primer, 2 μL of cDNA and 2.5 U of *HotStartTaq* DNA polymerase (Qiagen, Courtabœuf, France). The thermocycler profile was 15 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 45 °C and 2 min at 60 °C. For semi-nested PCR (snPCR), the reaction was achieved on 1 μL of the first amplification using 1.25 U of DNA polymerase under the same experimental procedures.

The PCR products were analysed on BET-stained agarose gels.

2.5 Sequencing of the amplicons

PCR products were purified by silica-based purification (Qiaquick, Qiagen). The sequences of the resulting amplicons were checked by sequencing with a BigDye terminator v3.1 kit (Applied Biosystems, Courtabœuf, France) on an ABI Prism 3140 automated sequencer (Applied Biosystems).

The amplicons generated with tailed primers were sequenced using primers M13Fwd and M13Rev (Table 1). The other ones were sequenced using PCR primers.

2.6 Assay sensitivity

The sensitivity of the assay was measured by using supernatants of cell cultures infected by PV-1 strain Sabin and EV-7 strain Wallace. The titers of these supernatants, expressed as $TCID_{50}$ per mL, were determined according to the WHO standard protocol (Anonymous, 2004). Serial 4-fold dilutions of the supernatants were made in cell culture medium. RNA was extracted and subjected to RT and PCR as exposed in sections 2.3 and 2.4.

3. Results

3.1 Sequence analysis and primer design

In order to amplify successfully regions located in different parts of the genome of human enteroviruses, generic primers were designed to target genomic sequences that encode conserved peptidic motifs in the viral polyprotein (Figure 1B).

In the P1 region, two conserved amino acid sequences were identified on both sides VP1. The sense primer AMTH targeted a region located ~ 270 nt upstream the VP1-encoding sequence. This region was also targeted by the primer 224 in a previously reported snPCR

assay (Nix et al., 2006); nevertheless, compared to the Nix' primer, the primer AMTH was slightly modified to take into account particular amino acidic sequences featured by several viruses, such as EV-13, HEV-69 and viruses belonging to the HEV-D species. In the P2 region of the genome, the primers targeted sequences encoding conserved cleavage sites of the polyprotein, at the 2A-2B and 2C-3A junctions. In the P3 region, the primers targeted highly conserved sequences in the 3C and 3D regions.

Consensus degenerate primers were designed by back-translating the conserved peptidic patterns into nucleotidic sequences. Deoxyinosine was introduced at the positions displaying 4-fold codon degeneracy. In order to simplify the sequencing step, the PCR primers were tagged with commonly-used M13 forward and reverse sequences to produce M13-labelled PCR products. Labelling of PCR products allowed the utilisation of identical primers for the sequencing of amplicons produced with different primer pairs (Bailly et al., 2000).

3.2 Assay evaluation

In a first time, the new primers pairs were tested on PV-1 and EV-7 supernatants whose titers was determined to be $10^{8.4}$ and $10^{8.0}$ $TCID_{50}$ per mL, respectively. Following the RT step with random primers performed on viral RNA extracted from undiluted supernatants, the primer pairs J-AMTH/J-GDCL, J-HEV.2AB.d/J-HEV.2C3A.c and J-HEV.2C.d1/J-HEV.3D.c2 produced detectable gel bands (Figure 2). The amplicons were successfully sequenced using M13 primers without gel extraction.

The same assay was then performed on PV-1 and EV-7 RNA extracted from diluted supernatants in order to determine the sensitivity of each primer pair (Figure 2 and Table 2).

On PV-1 RNA, the P2-targeting pair J-HEV.2AB.d/J-HEV.2C3A.c was able to generate amplicons for supernatants displaying a titer $\geq 10^{5.4}$ $TCID_{50}$ per mL whereas J-HEV.3C.d1/J-HEV.3D.c2 (P3 region) required titer $\geq 10^{6.0}$ $TCID_{50}$ per mL. The P1-targeting pair J-AMTH/J-GDCL was found to be poorly sensitive, requiring a titer $\geq 10^{7.0}$ $TCID_{50}$ per mL for positive results.

In an attempt to improve the sensitivity of the PCR assays, reactions were performed using the same primers deprived of M13 tags. In the P1 region, the new primers (called AMTH and GDCL) improved the sensitivity, getting a positive result for supernatants with titer $\geq 10^{6.0}$ $TCID_{50}$ per mL (Figure 2). Therefore, only the untagged primer pairs AMTH/GDCL was used to amplify the P1 region of the genome in the subsequent experiments. In contrast, in the P3 and P2 regions of the genome, achievement of the PCR with untagged primer pairs (HEV.3C.d1/HEV.3D.c2 and HEV.2AB.d/HEV.2C3A.c, respectively) did not improve dramatically the sensitivity of the assay (Table 2): in the P3 region, the untagged primers led to a slight improvement of the PCR sensitivity ($10^{5.4}$ instead of $10^{6.0}$); in the P2 region of the genome, no improvement was observed by using untagged primers.

In order to improve the sensitivity of the method, snPCR were assayed in the three regions of the genome. In the P1 region, the primers 224 and 222 previously designed by Nix *et al.* (Nix et al., 2006) were used; in the P3 region of the genome, a new primer (J-HEV.3C.d2) was designed. The P1- and P3-targeting primers were found to be able to generate detectable amplicons for supernatants featuring titer as low as $10^{3.0}$ $TCID_{50}$ per mL (Figure 2). In contrast, improvement of the sensitivity of the assay by snPCR in the P2 region was unsuccessful: all the primers designed in this region failed to achieve an amplification leading to gel bands suitable for direct sequencing (data not shown).

Sensitivity assays achieved with the EV-7 supernatant gave similar sensitivity values (data not shown).

In a second step, the assay was tested on 90 randomly-chosen prototype strains and field isolates available in the laboratory. These strains belonged to 39 different serotypes representative of the five human enterovirus species (Table 3). The assay succeeded in amplifying the P1 and the P3 regions for all the 90 tested strains either by RT-PCR (62 isolates of 90) or by RT-snPCR. For most of them (65 isolates of 90), the P2 region was also amplified by RT-PCR. In all cases, the PCR and/or snPCR assays led to gel-bands that were efficiently sequenced without gel extraction step.

4. Discussion

Human infections with enteroviruses are very frequent. Recombination events between different human enteroviruses are known to be very common, giving rise to a wide range of genotypes, of which some can exhibit threatening pathogenic properties. For this reason, many laboratories are involved worldwide in the surveillance of the human enterovirus circulation, particularly in countries with insufficient vaccine coverage against poliomyelitis. The surveillance, based on isolation of field strains, implies not only the determination of the serotype of these viruses (either by seroneutralization or by sequencing the capsid-encoding region), but also the determination of sequences of other genomic regions, in particular those of the non-structural moiety. Large-scale sequencing of circulating human enteroviruses, both in their structural and non-structural regions, is expected to provide new insights to understand better enterovirus genetics and to evaluate the possibility of emergence of new genotypes with increased pathogenic power.

The aim of this study was to develop a convenient method allowing the sequencing of regions distributed in the entire ORF of the genome of human enterovirus strains isolated in cell cultures. For this purpose, generic primers were designed by back-translating genomic sequences that encode peptidic patterns highly conserved among the five human enterovirus species. Some of the identified regions have already been targeted by other primers previously reported, such as CHR2, a primer targeting the 2C-3A junction of EV (Kottaridi et al., 2007), or the primers EUC12-a, -b and -c designed to partly sequence the 3D-coding region of HEV-B (Oprisan et al., 2002). After back-translation, the linear increase of the primer degeneracy level can be detrimental to amplification sensitivity due to the geometric increase in combinatory possibilities (Rossolini et al., 1994); for that reason, deoxyinosine was introduced in the primers at the nucleotidic positions displaying 4-fold degeneration.

Compared to the pair AMTH/GDCL, the lack of sensitivity of the primer pair J-AMTH/J-GDCL observed in the pilot experiments with PV-1 and EV-7 could be explained by the interference of the M13 tags, which could inhibit the reaction by steric hindrance or by creating fortuitous secondary structures. In contrast, untagged primers did not improve drastically the sensitivity of the P2- and P3-targeting assays. These results illustrate the fact that the influence of the primer tails on PCR yield is not clear, as already observed (Afonina et al., 2007).

For viral strains with titer $\geq 10^{6.0}$ $TCID_{50}$ per mL, the three amplicons obtained after the first round of PCR represented ~ 4,100 nt, *i.e.* more than 50 % of the whole genomic sequence. In the P1 region of the genome, the AMTH/GDCL PCR product encompassed the full VP1 gene, providing more complete information than the previously reported pan-human enterovirus VP1-targeting assays (Caro et al., 2001; Nix et al., 2006), designed to identify the serotype of field or clinical strains.

When grown in cell cultures, some human enteroviruses can display a titer lower than $10^{6.0}$ $TCID_{50}$ per mL. To make possible the typing of such viruses, snPCR were developed to increase the sensitivity of the assays. snPCR in the P1 and P3 regions were shown to be able to amplify sequences of virus with titer as low as $10^{3.0}$ $TCID_{50}$ per mL, giving rise to products covering about one quarter of the viral genome. In spite of the absence of snPCR in the P2 region, the majority of the tested strains grown in cell cultures were successfully amplified in this region by the single PCR with the primers J-HEV.2AB.d/J-HEV.2C3A.c.

Compared to other known methods developed to sequence genomic regions of human enteroviruses, this new assay has several advantages. First, contrary to other methods that require a specific primer set for each species (Iturriza-Gomara et al., 2006; Junttila et al., 2007), this assay succeeded in amplifying genome of field strains belonging to the five human enterovirus species. Second, the use of random primers for the RT reaction made it possible to reduce the workload, the same RT product serving as matrix in the three following PCR assays. Third, the purity of the amplicons and their sizes, lower than 1,500 bp, allowed the determination of overlapping sense and anti-sense sequences in a single round of sequencing without the need for gel-extraction.

In summary, a convenient method was developed for the determination of sequences of P1, P2 and P3 regions of the genome of human enteroviruses isolated in cell cultures. This method may be extremely useful for laboratories involved in the enterovirus survey, to type circulating viruses and to study the phenomenon of recombination between enteroviruses.

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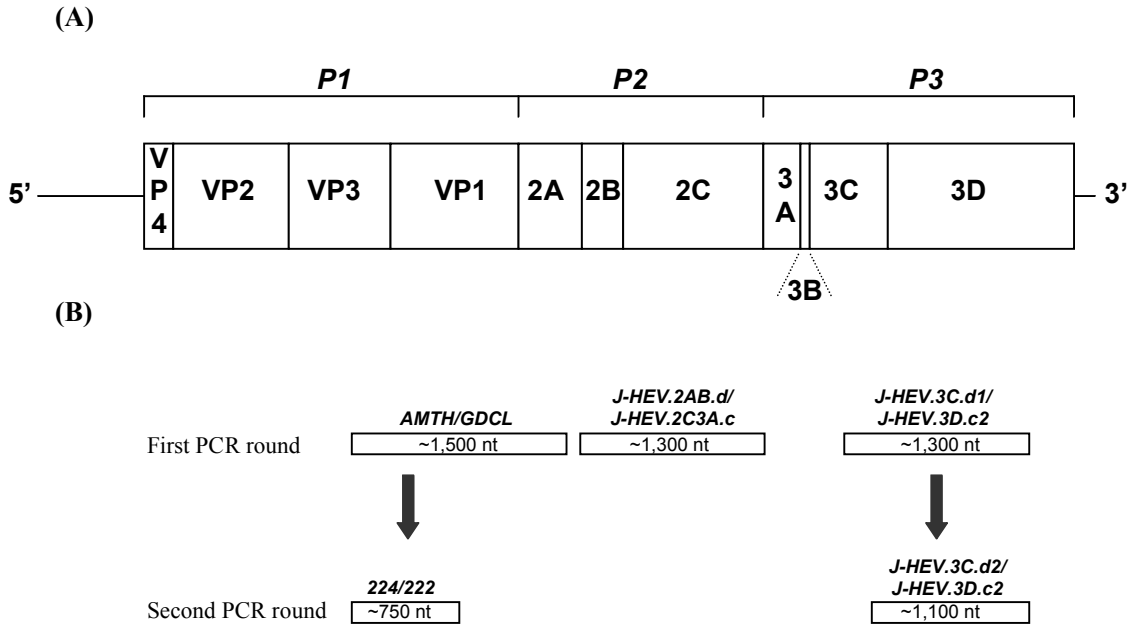
Figure captions

Figure 1. Schematic representation of the locations of the primers used in the RT-PCR and RT-snPCR. (A) Scheme of the organization of the human enterovirus genomic RNA. Boxes represent the structural and non-structural protein-encoding sequences of the unique ORF. 5'- and 3'-UTR are represented by lines. (B) Amplicons generated by RT-PCR and snPCR.

Figure 2. Sensitivity of the different RT-PCR and RT-snPCR on PV-1 RNA extracted from 4-fold serial dilutions. The same DNA ladder was used on each gel; the scale, expressed in bp, is indicated on the top left gel.

Figure(s)


(A)
P1
P2
P3
5'
VP4
VP2
VP3
VP1
2A
2B
2C
3A
3B
3C
3D
3'
(B)
AMTH/GDCL
J-HEV.2AB.d/
J-HEV.2C3A.c
J-HEV.3C.d1/
J-HEV.3D.c2
First PCR round
~1,500 nt
~1,300 nt
~1,300 nt
224/222
J-HEV.3C.d2/
J-HEV.3D.c2
Second PCR round
~750 nt
~1,100 nt

Figure(s)

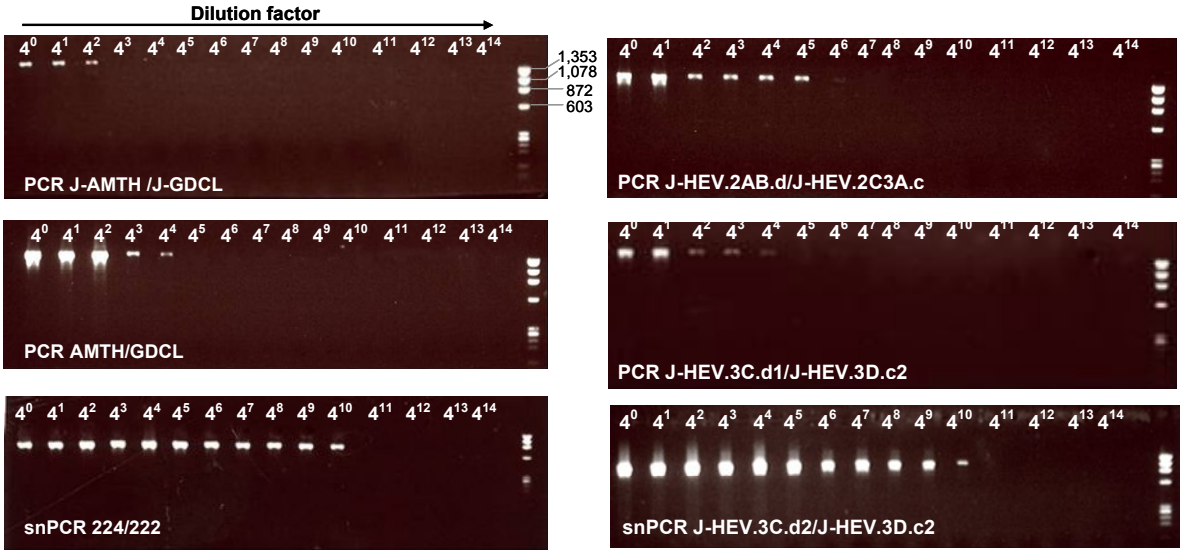

Dilution factor
4^0 4^1 4^2 4^3 4^4 4^5 4^6 4^7 4^8 4^9 4^10 4^11 4^12 4^13 4^14
1,353
1,078
872
603
PCR J-AMTH /J-GDCL
PCR J-HEV.2AB.d/J-HEV.2C3A.c
PCR AMTH/GDCL
PCR J-HEV.3C.d1/J-HEV.3D.c2
snPCR 224/222
snPCR J-HEV.3C.d2/J-HEV.3D.c2

Table 1. Primers used in this study.

Name	5'→3' sequence [a]	Amino-acid motif	Genome position [b]
J-AMTH	*GTAAAACGACGGCCAG*GCIATGYTIGSIACICAYVT	AML(G/A)TH(V/I/L/M)	2204-2223
AMTH	GCIATGYTIGSIACICAYVT	AML(G/A)TH(V/I/L/M)	2204-2223
J-GDCL	*CAGGAAACAGCTATGAC*ARNABNCCNCCRCARTCNCC	GDCGG(I/V)L	3714-3695
GDCL	ARNABNCCNCCRCARTCNCC	GDCGG(I/V)L	3714-3695
224 [c]	GCIATGYTIGGIACICAYRT	AMLGTH(I/L/M)	2204-2223
222 [c]	CNCCNGGNGGNAYRWACAT	M(F/Y)(I/V)PPG(A/G)	2960-2942
J-HEV.2AB.d	*GTAAAACGACGGCCAG*GAIGYIATGGARCARGG	(D/E)(A/V)MEQG	3809-3825
HEV.2AB.d	GAIGYIATGGARCARGG	(D/E)(A/V)MEQG	3809-3825
J-HEV.2C3A.c	*CAGGAAACAGCTATGAC*GGICCYTGRAAIARIGCYTC	EALFQGP	5106-5087
HEV.2C3A.c	GGICCYTGRAAIARIGCYTC	EALFQGP	5106-5087
J-HEV.3C.d1	*GTAAAACGACGGCCAG*AAYGARAARTTYMGIGAYAT	NEKFRDI	5666-5685
HEV.3C.d1	AAYGARAARTTYMGIGAYAT	NEKFRDI	5666-5685
J-HEV.3C.d2	*GTAAAACGACGGCCAG*GCIGGICARTGYGGIGGIRT	AGQCGG(V/I)	5858-5877

J-HEV.3D.c2	*CAGGAAACAGCTATGAC*ACRTCRTCICCRTAIGCIAYCAT	M(I/V)AYGDDV	6966-6944
HEV.3D.c2	ACRTCRTCICCRTAIGCIAYCAT	M(I/V)AYGDDV	6966-6944
M 13Fwd	GTAAAACGACGGCCAG		
M 13Rev	CAGGAAACAGCTATGAC		
heptaN	NNNNNNN		

[a] in tailed primers, whose names begin by "J-", the M13 tags are indicated in italic.

[b] relative to PV-3 strain Leon.

[c] according to Nix et al., 2006.

Table 2. Sensitivity of the RT-PCR and RT-snPCR.

Targeted region of the viral genome	Assay	Primer pair	Sensitivity[a] ($TCID_{50}$ per mL)
P1	PCR	J-AMTH/J-GDCL	$10^{7.2}$
	PCR	AMTH/GDCL	$10^{6.0}$
	snPCR	224/222	$10^{3.0}$
P2	PCR	J-HEV.2AB.d/ J-HEV.2C3A.c	$10^{5.4}$
	PCR	HEV.2AB.d/ HEV.2C3A.c	$10^{5.4}$
P3	PCR	J-HEV.3C.d1/ J-HEV.3D.c2	$10^{6.0}$
	PCR	HEV.3C.d1/ HEV.3D.c2	$10^{5.4}$
	snPCR	J-HEV.3C.d2/ J-HEV.3D.c2	$10^{3.0}$

[a] For each primer pair, the sensitivity was defined as the titer of the most diluted supernatant allowing the generation of an amplicon.

Table(s)

Serotype	Number of isolates	P1 and P3 genomic parts: number of isolates efficiently amplified by PCR without the need for snPCR [a]	P2 genomic part: number of isolates efficiently amplified by PCR
HEV-A			
CV-A2 strain Fleetwood	1	1	1
CV-A3 strain Olson	1	1	1
CV-A6 strain Gdula	1	1	1
CV-A10*	2	2	2
CV-A10 strain Kowalik	1	1	1
CV-A12 strain Texas-12	1	1	1
CV-A14 strain G-14	1	1	1
CV-A16 strain G-10	1	1	1
HEV-71*	1	1	1
HEV-B			
CV-A9*	1	1	1
CV-B1*	1	1	1
CV-B4*	2	1	1
CV-B5*	1	1	1
EV-1*	1	1	1

EV-3*	4	3	3
EV-6*	4	1	2
EV-7*	6	3	3
EV-7 strain Wallace	1	1	1
EV-8*	2	1	1
EV-9*	1	1	1
EV-11*	4	1	2
EV-12*	3	1	1
EV-13*	4	3	3
EV-19*	3	1	1
EV-20*	3	1	1
EV-21*	3	3	3
EV-29*	2	1	1
EV-30*	1	1	1
EV-33*	2	1	1
HEV-69*	1	1	1
HEV-74*	2	2	1
HEV-C			
CV-A11*	3	3	3
CV-A13*	8	5	6
CV-A17*	2	2	2
CV-A20*	4	2	2
CV-A21*	2	1	1
CV-A24*	4	3	4

HEV-D			
HEV-70*	2	2	2
PV			
PV-1 strain Sabin	1	1	1
PV-2 strain Sabin	1	1	1
PV-3 strain Sabin	1	1	1
Total	90	62	65

Table 3. HEV strains tested for amplification. Prototype viruses are listed with their names. Asterisks indicate unpublished field strains.

[a] All the isolates which have not been amplified efficiently by PCR in the P1 and P3 parts of their genome were amplified successfully by snPCR.