

Identification of 5' capped structure and 3' terminal sequence of hepatitis E virus isolated from Morocco

Guo-Bing Chen, Ji-Hong Meng

Guo-Bing Chen, Ji-Hong Meng, Department of Microbiology and Immunology, Southeast University School of Medicine, Nanjing 210009, Jiangsu Province, China

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Correspondence to: Professor Ji-Hong Meng, Department of Microbiology and Immunology, Southeast University School of Medicine, Nanjing 210009, Jiangsu Province, China. jihongmeng@263.net

Telephone: +86-25-3272454

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Abstract

AIM: To examine 5' and 3' terminal sequences of hepatitis E virus (HEV) isolated from Morocco, to confirm 5' methylated cap structure of the genome, and to investigate whether the 3' UTR can be used to distinguish HEV genotypes instead of HEV complete genome sequence.

METHODS: RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) was employed to obtain the 5' and 3' terminal sequences of HEV Morocco strain. The 3' UTR sequence of the Morocco strain was compared with that of the other 29 HEV strains using the DNASTar software.

RESULTS: The 5' PCR product was obtained only from the RLM-RACE based on the capped RNA template. The 5' UTR of the Morocco strain had 26 nucleotides, and the 3' UTR had 65 nucleotides upstream to the polyA. The 5' UTR between HEV strains had only point mutations of nucleotides. The phylogenetic tree based on the sequences of 3' UTR was not the same as that based on the complete sequences.

CONCLUSION: The genome of HEV Morocco strain was methylated cap structure. The 3' terminal sequence can not be used for distinguishing HEV genotype for all HEV strains in place of the whole HEV genome sequence.

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INTRODUCTION

Hepatitis E virus (HEV) is an enterically transmitted agent that causes epidemic and sporadic cases of hepatitis predominantly in developing countries of Asia, Africa and North America^[1]. The disease generally affects young adults and has a high mortality rate, up to 20%, in infected pregnant women. Success in cloning and sequencing of the HEV genome allowed the elucidation of the HEV genetic organization^[2]. The HEV genome is a positive-sense, single-stranded, polyadenylated RNA of approximately 7.2 kb containing three open reading frames (ORFs). ORF1, located at the 5' end of the genome, is

about 5 kb in length and encodes for a putative nonstructural polyprotein that contains motifs characteristic for methyltransferase, papain-like protease, RNA helicase, and RNA-dependent RNA polymerase domains. ORF2 is about 2 kb in length and encodes for the structural protein(s). The small ORF3 of only 369 nucleotides overlaps ORF1 and ORF2 and encodes for a protein of unknown function^[3].

The genomes of several HEV strains from Asia and North America have been sequenced in their entirety^[4]. Partial sequences are also available for other strains from some of these geographic regions. In Africa, the HEV virus has been identified from Morocco, Tunis, Algeria, Chad, Egypt, Nigeria and Namibia^[5-10]. However, only a few short isolated nucleotide sequences from African strains of HEV were available for analysis. In 1997, Chatterjee *et al.* isolated an HEV strain in Morocco^[5]. In 1999, Meng *et al.* obtained the nucleotide sequences of HEV Morocco strain that spanned the extreme 3' terminal region of ORF1, full length ORF2 and ORF3, and a part of the 3' noncoding region^[11,12]. Although more similar to the Asian strains than to the Mexico strain, partial HEV sequences from Morocco strains were, nonetheless, distinct from all known Asian strains. It is urgent to obtain the complete sequence of the Morocco strain to elucidate its biological function.

Although the genomic coding regions of several HEV strains have been sequenced completely, the sequence of the 5' UTR has been reported only for a limited number of HEV strains^[13]. The 5' UTR of HEV strains were usually determined either by cDNA cloning and sequencing or by classical 5' rapid amplification of cDNA ends (RACE)^[13]. However, there still exists the possibility that the 5' UTR sequences are not complete because neither method has a control to detect premature termination of cDNA synthesis. Furthermore, by coupling a reverse transcription polymerase chain reaction (RT-PCR) assay with immune-capture of genomic RNA based on the ability of a monoclonal antibody to recognize 7-methylguanosine showed that the genomic RNA of HEV is capped^[14]. It is not clear how this structure will have affected previous assays used to determine the 5' terminal nucleotides. For getting the complete sequence of HEV Morocco strain, we employed a RLM-RACE technique to obtain the 5' and 3' terminal sequences in our study, and confirmed that the genome of Morocco strain was capped.

Furthermore, how to distinguish the genotypes of HEV is still an issue. Based on the complete sequence analysis, Burma-1, Burma-2, China-1 to -6, Pak-1, Pak-2, India-1 to -4, Nepal and Morocco strains are included in genotype I; Mexico strain belongs to genotype II; Genotype III holds US-1, US-2, US-SW, Japan-1 to -7, Japan-SW and Canada-SW; China-T1 belongs to genotype IV^[15-17]. Considering the difficulties in obtaining the complete sequences of each strain, we attempted to evaluate whether the 3' UTR can be used to analyze the HEV genotype in this study.

MATERIALS AND METHODS

Sample

The sample was obtained from Professor Pillot, Pasteur Institute, France, and treated as described in references 16 and

17. Aliquots of virus stock were prepared and stored at -70 °C.

Reagents

HEV RNA was extracted with TRIzol (GIBCO, USA). The 5' and 3' RLM-RACE was carried out with the GeneRacer kit (Invitrogen, USA). The nested PCR was carried out with high fidelity system DNA polymerase (Roche, USA). PCR products were purified with QIAquick PCR purification kit (Qiagen, German).

Primers

5' RACE primers: Two RNA 5' -adaptor primers supplied in the GenRacer kit were used as forward primers. The sequences of the reverse primers were: 5' -AGA AAA GGC CTA ACC ACC ACA GCA TTC G-3' (outer reverse primer) and 5' -CTA AAG CAG CCT GCT CAA TAG CAG TAG-3' (inner reverse primer).

3' RACE primers: Sequences of the forward primers were: 5' -GTT GTC TCA GCC AAT GGC GAG C-3' (outer forward primer) and 5' -AAG ATG AAG GTG GGT AAA ACT CGG GAG-3' (inner forward primer). Two RNA 3' -adaptor primers supplied in the GenRacer kit were used as reverse primers.

GenBank accession number and software

The GenBank accession number of 3' UTR sequence of HEV Morocco strain was AY220474. The 5' and 3' UTR sequence of Morocco strain were compared with that of other 29 strains, respectively, by the MegAlign in DNASTar software (DNASTAR Inc, USA). The phylogenetic trees of 3' and complete HEV sequences were also made with MegAlign in DNASTAR. The GenBank accession numbers of these sequences were: Jap-SW (AB073912)^[18], Japan-1 (AB074918), Japan-2 (AB074920)^[19], Japan-3 (AB089824)^[20], Japan-4 (AP003430)^[21], Japan-5 (AB074915), Japan-6 (AB074917)^[19], Japan-7 (AB080575)^[22], Nepal (AF051830)^[23], US-1 (AF060668), US-2 (AF060669)^[24,25], US-SW (AF082843)^[26], Canada-SW (AY115488)^[27], India-1 (X98292), India-2 (AF459438)^[28], India-3 (AF076239)^[29], India-4 (X99441), Pak-1 (M80581)^[30], Pak-2 (AF185822)^[31], Burma-1 (M73218)^[3], Burma-2 (D10330)^[32], China-1 (L25547), China-2 (L25595)^[33], China-3 (L08816), China-4 (M94177)^[34], China-5 (D11092)^[35], China-6 (D11093), Mexico (M74506)^[4] and China-T1 (AJ272108)^[36]. The RNA secondary structure was reconstructed with RNAstructure software (Isis Pharmaceuticals).

Extraction of HEV RNAs

One hundred microliters of the filtered supernatant of the Morocco strain was mixed with 400 µL of TRIzol reagent. The mixture was homogenized and incubated for 5 min at room temperature. One hundred microliters of chloroform was added and the mixture was vigorously shaken for 15 s and incubated at room temperature for 3 min. After centrifugation at 12 000 g for 15 min at 4 °C, the aqueous phase was transferred to a fresh microfuge tube. The RNA from the aqueous phase was precipitated by incubating with an equal volume of isopropyl alcohol and 1 µL of glycogen (20 mg/mL) at room temperature for 15 min, and centrifuging at 12 000 g at 4 °C for 15 min. After removing the supernatant, the RNA pellet was washed once with 800 µL of 750 mL/L ethanol and centrifuged again, dried, and then dissolved in 20 µL of diethylpyrocarbonate treated water.

5' RLM-RACE

The 5' RACE was carried out with the GeneRacer kit following the manufacture's instructions. Briefly, 14 µL of extracted RNA was treated with calf intestinal phosphatase (CIP) in a total 20 µL reaction mixture containing 2 µL of 10× CIP buffer

and 2 µL of CIP for 1 h at 50 °C. After extracted with phenol/chloroform, RNA was resuspended in 6 µL of nuclease-free water. The 6 µL of CIP-treated RNA was treated with tobacco acid pyrophosphatase (TAP) in a 10 µL of reaction mixture containing 1 µL of 10× TAP buffer and 2 µL of TAP for 1 h at 37 °C. Again after extracted with phenol/chloroform, the CIP/TAP-treated RNA was resuspended in 6 µL of nuclease-free water and ligated to 250 ng of RNA adaptor by T4 RNA ligase in a 10 µL reaction mixture for 1 h at 37 °C. Then the CIP/TAP/Ligated-RNA was extracted with phenol/chloroform and resuspended in 20 µL nuclease-free water and reserved at -70 °C. Another 6 µL non-treated RNA was ligated to 250 ng of RNA adaptor directly by T4 RNA ligase as a control. Ten microliters of the ligated RNA or control RNA was used as a template to synthesize cDNA with AMV reverse transcriptase for 1 h at 42 °C. The outer reverse primer was used to prime the cDNA synthesis. The cDNA was then amplified by nested PCR with high fidelity system DNA polymerase. The PCR reaction mixture was incubated for 2 min at 94 °C followed by 35 amplification cycles, comprising denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s and extension at 72 °C for 30 s. The reaction was extended for another 7 min at 72 °C to insure the full extension. Based on our former work (data not shown), the expected size of the final PCR product was 100 bp or greater. PCR products were analyzed on 20 g/L agarose gel.

3' RACE

The 3' RACE was carried out with the GeneRacer kit following the manufacture's instructions. Ten microliters of the HEV RNA was used as a template to synthesize cDNA with AMV reverse transcriptase for 1 h at 42 °C. The oligo (dT)-adaptor primer supplied with the kit was used to prime the cDNA synthesis. The cDNA was then amplified by nested PCR with high fidelity system DNA polymerase. The PCR reaction mixture was incubated for 2 min at 94 °C, followed by 35 amplification cycles comprising denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, and extension at 72 °C for 30 s. The reaction was extended for another 7 min at 72 °C to ensure the full extension. Based on our former work (data not shown), the expected size of the final PCR product was 98 bp or greater. PCR products were analyzed on 20 g/L agarose gel.

Sequence analysis and comparison

PCR products were purified with QIAquick PCR purification kit and sequenced. The 5' and 3' UTR sequence of Morocco strain were compared with that of other 29 strains, respectively, using DNASTar software. The RNA secondary structure was reconstructed with RNAstructure software. The phylogenetic trees of 3' and complete HEV sequences were also made with DNASTAR.

RESULTS

5' UTR

As shown in Figure 1A, bands of the expected size were obtained. The band was obtained in 5' RLM-RACE reactions only when the template was CIP/TAP treated RNA.

The 5' terminus of Morocco had 26 nucleotides preceding the predicted start codon, including the 7-methylguanosine. The sequence of PCR product was: GGC AGA CCA CAT ATG TGG TCG ATG CC. A comparison of the 5' UTR sequence of 30 HEV strains was made as shown in Figure 2: there was a "T" at the position 4 preceding the predicted start codon in Morocco and other 25 strains while a "C" was found in other four strains; a "G" at the position 9 in the Morocco and other 28 strains turn out to be a "T" in US-SW strain; an "A" at the position 16 in Morocco and other 20 strains was replaced by a "G" or "C" in other nine strains; an additional

"A" at the terminus of eight strains except Morocco strain; and additional 9 nucleotides in the US-2 terminus.

As shown in Figure 3, the 26 nucleotides of 5' terminus could form a putative stem-loop structure. All single-nucleotide differences occurred within that loop structure.

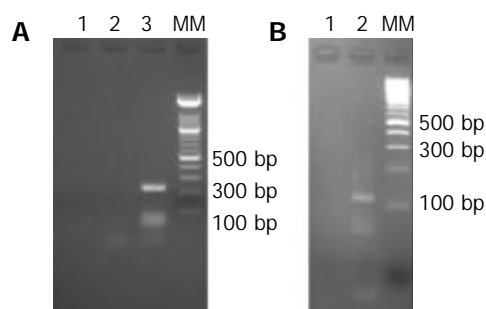


Figure 1 RT-PCR products of 5' and 3' RNA of HEV Morocco strain. A: Products of 5' RLM-RACE; Lane 1: negative control without template RNA; Lane 2: RNA template without CIP/TAP treated; Lane 3: RNA template treated with CIP and TAP. B: Products of 3' RACE; Lane 1: negative control without template RNA; Lane 2: products of 3' RACE; MM: 100 bp ladder.

Sequence Name	< Pos =	35	30	25	20	15	10	5	1
Burma-1 [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
Burma-2 [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
Canada-3W [3]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
China-1 [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
China-2 [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
China-3 [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
China-4 [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
China-5 [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
China-6 [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
China-T1 [4]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
India-1 [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
India-2 [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
India-3 [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
India-4 [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
Japan-1 [3]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
Japan-2 [3]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
Japan-3 [3]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
Japan-4 [3]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
Japan-5 [4]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
Japan-6 [4]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
Japan-7 [4]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
Jap-3W [3]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
Mexico [2]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
MOROCCO [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
Nepal [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
Pak-1 [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
Pak-2 [1]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
US-1 [3]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
US-2 [3]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						
US-3W [3]		-----	<u>GGCAGACCACATATGTGGTCGATGCCATG</u>						

Figure 2 Comparison of sequence in the 5' UTR of different HEV strains. The translation initiation codon is underlined. The number in brackets following the strain name is the genotype designation. Changes from the consensus sequence are boxed.

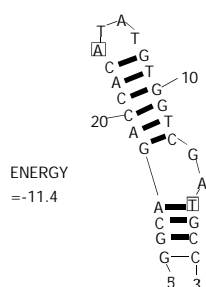


Figure 3 RNA secondary structure of HEV 5' terminal sequence. Changes among HEV strains are boxed.

3' UTR

As shown in Figure 1B, 3' RACE bands of the expected size were obtained. The 3' terminus of Morocco had 65 nucleotides upstream of the polyA (the GenBank accession is AY220474). The sequence of 3' UTR was: TTT ATT TGC TTG TGC CCT CCT TCT TTC TGT TGC TTA TTT CTC TTT TCT GCG TTT CGC GCT CCC TG. The comparison of the 3' UTR sequence with the corresponding regions of other 29 HEV strains from different parts of the world revealed that the Morocco strain was most similar to the genotype 1 strains (92.3-97.4%) (Table 1). The 3' UTR nucleotide sequences of the HEV genotypes II, III and IV strains shared a lower similarity with that of Morocco strain: 80%, 65.6-83.1% and 84.6-89.2%, respectively. The phylogenetic trees also showed that the HEV Morocco strain was grouped together with all genotype I strains as a separate branch (Figures 4 and 5). Based on the 3' UTR analysis, Japan-5, -6, -7 belonged to genotype III. But according to the complete sequence analysis, they were included in genotype IV.

Table 1 Percent identity of 3' UTR of HEV genotypes (%)

	I	II	III	IV
Mor	92.3-97.4	80	65.6-83.1	84.6-89.2
I	92.3-100	74.4-81.5	25.6-84.6	79.5-90.8
II	-	100	37.5-71.8	55.1-83.8
III	-	-	75.9-94.4	41.4-80.0
IV	-	-	-	77.9-94.3

I, II, III, IV: HEV Genotype; Mor: HEV Morocco strain; -: blank well.

DISCUSSION

Variations in the length of the 5' UTR have been observed. The Burma strain was reported to have 27 nucleotides in its 5' UTR, compared with 35 nucleotides in the 5' UTR of US-2 and 26 nucleotides in the most of other strains^[13]. It remains to be determined if the extra nucleotides are really present in functional genomes. Maybe they just represented artifacts generated because the presence of a cap structure was not recognized at the time some of those strains were sequenced, or because of the additional "A" with the non-proofreading DNA polymerase in the PCR action. What was more interesting, the US-2 strain had additional nine nucleotides at the 5' terminus. They were included in the GenBank sequence but had not been discussed elsewhere, so they were difficult to evaluate. Except the US-2, all HEV strains had a comparable 5' UTR sequence with only point change from each other, and the single-nucleotide differences occurred within the loop structure. This structure may play a very important role either in replication or translation. It needs to be verified experimentally.

The 5' UTR sequences obtained by the methods of cDNA cloning/sequencing or classical 5' rapid amplification of cDNA ends (RACE) may be incomplete because neither method has a control to detect premature termination of cDNA synthesis. The finding of a 7mGpppG cap allowed reevaluation of 5' UTR sequences with a modified RACE technique called RNA ligase-mediated RACE (RLM-RACE). In RLM-RACE, an RNA sample was first treated with calf intestinal phosphatase (CIP) to remove the 5' -phosphate from all RNA species except those with a cap structure. Tobacco acid pyrophosphatase (TAP) was then used to remove the cap structure from RNA, leaving a 5' -phosphate. Next, a synthetic RNA adaptor was ligated to the CIP/TAP treated RNA. Because the adaptor ligates only to RNA containing a 5' -phosphate, RLM-RACE ensures that cDNA was amplified from decapped RNA predominantly, starting at the ultimate 5' terminus. In 2001,

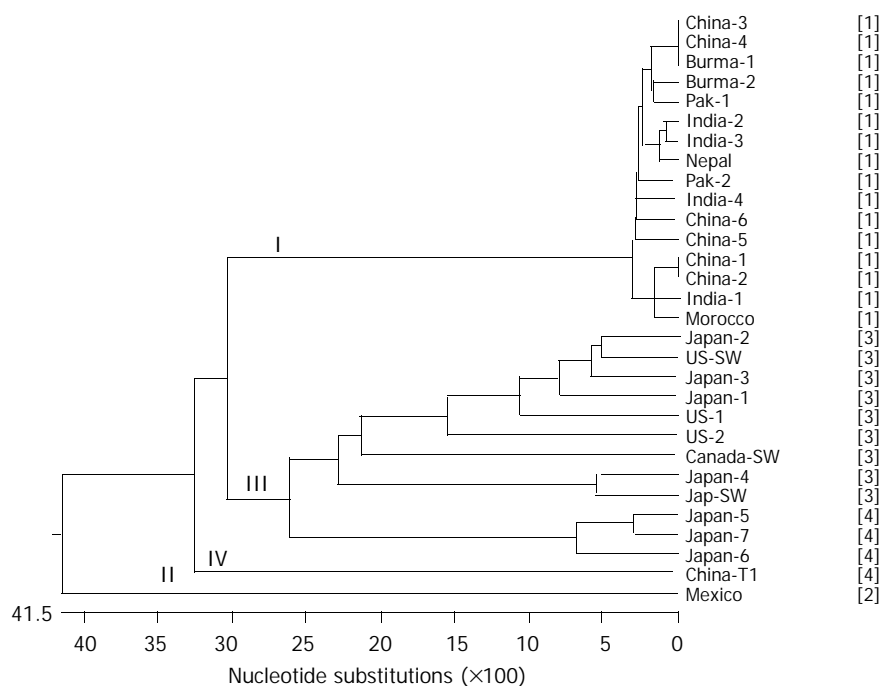


Figure 4 Proposed phylogenetic trees using 3' UTR sequences of Morocco and other 29 HEV strains. The Roman number in the tree represents the genotype designation based on the 3' UTR sequence. The number in brackets following the strain name represents the genotype designation based on the full genome sequence.

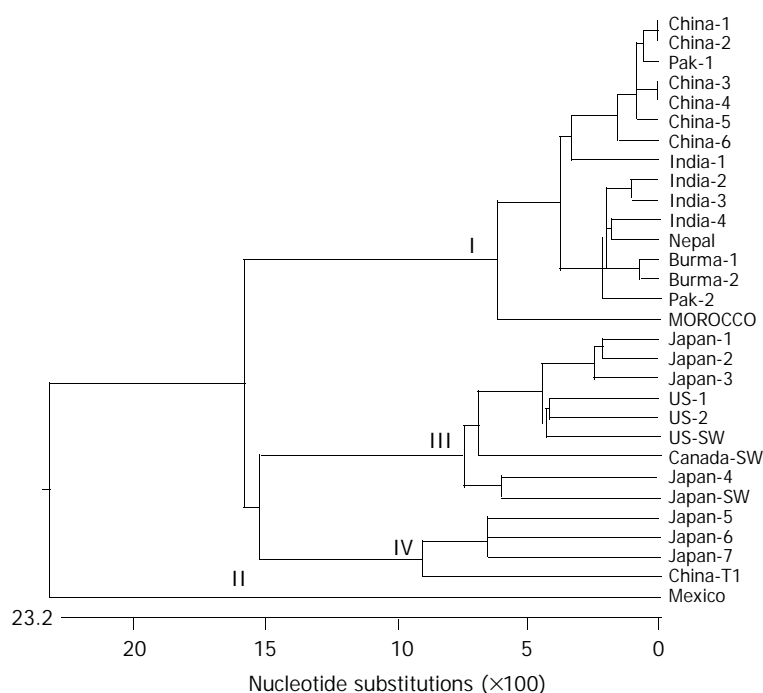


Figure 5 Proposed phylogenetic trees using full sequences of 30 HEV strains. The Roman number in the tree represents the genotype designation.

Zhang *et al.* used the RLM-RACE method to confirm the capped HEV genome and extended the 5' terminal sequence of Mexico and Pak-1 strains^[13]. In the current study to obtain the complete sequence of HEV Morocco strain, we used a sequence amplification procedure that required the presence of an internal pyrophosphatase sensitive linkage in the 5' termini of HEV genome. The results indicated that the Morocco strain was capped.

The comparison of the 3' UTR sequence with the corresponding regions of other 29 HEV strains and the phylogenetic trees of those complete sequences of 30 HEV strains revealed that the HEV Morocco strain was grouped together with all genotype

I strains as a separate branch. The distribution of 3' UTR of genotypes I, II and IV was as same as that obtained by the comparison of the complete genome sequence. However, three strains isolated from Japan, Japan-5, -6, -7, which were included in genotype IV according to complete sequences analysis, clustered together with the genotype III strains based on the 3' UTR sequences analysis. This suggests that the comparison of HEV 3' UTR sequence can not represent the divergence of complete genome sequence perfectly, although this region is regarded as containing the majority of genotype-specific nucleotide positions, and easily and efficiently to amplify. So, the 3' terminal sequence upstream the polyA can not be used

for phylogenetic genotyping analysis of the HEV genome optionally. Because of the difficulties to get the complete sequences, many scientists preferred to analyze the HEV genotypes based on the partial sequences they obtained. It remains uncertain whether the partial sequences can replace the complete genome in distinguishing the HEV genotypes. So, it is prudent to analyze the HEV genotypes based on complete sequences to date.

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