

Evidence for hepatitis E virus quasispecies

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The genetic diversity of hepatitis E virus (HEV) has been extensively analysed during the last decade. Most sporadic and epidemic HEV strains are distributed into genotypes or groups. Nevertheless, few studies have looked at the polymorphism of HEV strains isolated from a given outbreak. A serum bank collected in Tanefdour, Algeria, during an acute hepatitis epidemic (1986–1987), retrospectively confirmed as hepatitis E, was analysed. Of the 69 serum samples collected within an 8-week period, 23 were positive for both partial ORF1 (replicase gene) and ORF2 (capsid gene) sequences. Inter- and intra-patient diversities were assessed by RFLP, and by sequencing a 448 bp sequence corresponding to ORF2. RFLP analysis distinguished three profiles: A (18/23), B (3/23) and C (2/23). Most isolates (18/23) shared 99·7–100 % sequence identity and the remainder showed 1–1·3 % divergence. HEV intra-patient diversity was studied using 12 isolates (seven displaying the major RFLP profile and five displaying minor RFLP profiles). For 9 of 12 isolates, additional intra-patient heterogeneity was revealed by RFLP analysis of 100 clones from each isolate and sequence diversity ranging from 0·11 to 3·4 %. These data strongly support the quasispecies organization of HEV during epidemics and could explain the adaptable behaviour of the virus in the host–pathogen interrelations.

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INTRODUCTION

Hepatitis E virus (HEV) is a major cause of enterically transmitted acute hepatitis in developing countries. Large epidemics have been reported in Asia, Africa and Latin America following fecal contamination of drinking water resources. In industrialized countries, sporadic cases of hepatitis E have been reported, either imported by travellers from endemic areas or of unspecified native origin (Clemente-Casares *et al.*, 2003).

Although HEV strains belong to a single serotype, they display considerable genetic diversity according to the time and place of isolation. Different classification systems for HEV isolates have been proposed. According to the systems, they have been divided into four major genotypes (1 to 4) or at least nine groups (Schlauder & Mushahwar, 2001). These taxonomic systems are based on the full-length sequences of representative HEV strains isolated from epidemics (Aye *et al.*, 1992; Huang *et al.*, 1992; Tsarev *et al.*, 1992) or sporadic HEV isolates (Schlauder *et al.*, 1999;

Takahashi *et al.*, 2002). Partial sequencing of HEV genotype 1 strains revealed genetic changes with sporadic acute hepatitis E and inter-epidemic polymorphism (Shrestha *et al.*, 2004), but few studies have looked at the genetic diversity of HEV isolates within one epidemic. In India, analysis of the replicase gene of 17 HEV strains isolated from seven epidemics that occurred over a 17-year period (1976–1993), each epidemic being represented by one to five HEV isolates, showed only minor inter-epidemic genetic variation and no intra-epidemic diversity (Arankalle *et al.*, 1999). Analysis of ORF1 (replicase gene) and ORF2 (capsid gene) sequences of HEV stool isolates from three Indian epidemics that occurred over a 9-year period, each epidemic being represented by five HEV isolates, showed clear inter-epidemic diversity (3 % for ORF1 and 4 % for ORF2) and unexpected inter-patient variation within the same epidemic, suggesting either the co-circulation of closely related HEV strains or the emergence of quasispecies (Aggarwal *et al.*, 1999).

Quasispecies is a level of genomic diversity that characterizes RNA viruses in which the non-proofreading polymerase leads to error-prone replication, resulting in better environmental adaptability and capacity for rapid evolution during

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passage from host to host (Schneider & Roossinck, 2001). It has been described mainly in persistent virus infections, such as those due to human immunodeficiency virus type 1 and hepatitis C virus, during which virus populations develop a high degree of sequence variation within each infected individual (Sanchez-Palomino *et al.*, 1996). It is less common in acute self-limited virus diseases such as those caused by dengue virus (Wang *et al.*, 2002) or hepatitis A virus (Sanchez *et al.*, 2003).

To assess the genetic heterogeneity of the HEV strains involved in an outbreak, we carried out a retrospective analysis of both inter- and intra-patient diversity on 23 serum samples collected during a water-borne outbreak that occurred in 1986–1987 in eastern Algeria. Our results provide the first proof of the quasispecies nature of epidemic HEV.

METHODS

Background of the epidemic. A large jaundice epidemic occurred from March 1986 to January 1987 in a rural area of eastern Algeria. This epidemic was centred in Tanefdour, a village with 3119 inhabitants in which 247 cases were notified (Table 1). Attack rates were similar in males and females (7.4 and 8.4%, respectively) with a global fatality rate reaching 1.2% for the entire population and 8.7% among pregnant women. A population-based investigation showed that the consumption of spring water was associated with a significantly greater risk than the consumption of well water (Siriwardana *et al.*, 2003).

For 6 weeks in January and early February 1987, blood samples were collected from 69 jaundice patients. The patients were initially diagnosed with non-A, non-B virus water-borne hepatitis. Serum samples were stored at -20°C until HEV antibody testing (HEV EIA; Abbott). Of the 69 serum samples tested, 65 exhibited a strong reactivity for specific antibodies, with optical density/cut off (OD/CO) values over 2 for 62 of them and over 5 for three of them, confirming a recent HEV infection.

HEV genome amplification. Total RNA was extracted from the 69 serum samples using the QIAamp virus RNA mini kit (Qiagen) according to the manufacturer's instructions. Based on the sequence of the Burmese strain, oligonucleotides were designed to target the HEV ORF1 spanning nt 4412–4910 (replicase gene) and ORF2 nt 6653–7100 (capsid gene). Nested PCR was performed as described elsewhere (Tam *et al.*, 1991). Briefly, the RT-PCR step was performed in a GeneAmp 2400 thermocycler using the Titan One Tube RT (Boehringer Mannheim) as follows. After incubation at 50°C for 45 min, the first PCR amplification was carried out at 94°C 7 min, 45°C 45 s, 68°C 45 s for one cycle; 92°C 30 s, 45°C 45 s, 68°C 45 s for 39 cycles. Nested PCR was performed using 5 μl of the PCR product with *Pfu* polymerase (Stratagene) and the following conditions: 94°C 7 min, 50°C 45 s, 72°C 45 s for one cycle and 92°C

30 s, 50°C 30 s, 72°C 45 s for 39 cycles. Amplification products were analysed by conventional agarose gel electrophoresis. The specificity of amplimers was controlled using internal digoxigenin end-labelled oligonucleotide probes (Buisson *et al.*, 2000).

Molecular typing of the epidemic strain

Restriction endonuclease analysis. HEV genotyping was carried out on ORF1 and ORF2 nested PCR products by endonuclease restriction analysis as described previously (Gouvea *et al.*, 1998; Buisson *et al.*, 2000). Briefly, it was possible to distinguish between genotypes 1 and 2 by digestion of the ORF1 PCR product with *SmaI* and the ORF2 product with *KpnI* and *NotI*. The ORF2 product was also digested with *BsmI* to distinguish African genotype 2 strains from the Mexico prototype (Buisson *et al.*, 2000).

To assess the genetic heterogeneity of HEV isolates involved in this epidemic, the 448 bp ORF2 PCR fragment was subjected to RFLP analysis. Crude PCR products were digested in a final volume of 20 μl with 1.5 U *Fnu4HI* (New England Biolabs) for 2 h at 37°C . Digested products were initially analysed on a 3% agarose gel (2% LE agarose; Promega; 1% high resolution agarose; Sigma). Digested amplimers (6 μl) were loaded on a precast polyacrylamide gel and subjected to electrophoresis (as recommended by the supplier) in a GenPhor electrophoresis unit (GeneGel Excel 12.5/2; Amersham Biosciences). Separated products were silver stained (PlusOne silver staining kit; Amersham Biosciences). RFLP profiles were analysed using 1D Image analysis software (Kodak Digital).

The same procedure was used on the following cloning products. To study the diversity of the virus population, 12 isolates [seven randomly selected isolates with the most common RFLP profile (A) and five isolates displaying the other two RFLP profiles (B and C)] were cloned as described below.

Cloning and sequence analysis. To assess inter-patient diversity, the ORF2 products amplified from 23 of 69 samples were purified on Microspin 200-HR columns (Amersham Biosciences) and sequenced in both directions with the DTCS sequencing kit (Beckman Coulter) by using an automated DNA sequencer (CEQ 8000; Beckman Coulter).

Intra-patient diversity was evaluated by analysing the 12 isolates described previously. Firstly, amplified ORF2 products were treated with *Taq* polymerase (Promega) (30 min, 72°C) resulting in DNA 3' A ends then purified and cloned into pCR-Script SK(+) (Stratagene) or into pGEM-T (Rapid ligation kit; Promega) as recommended by the manufacturers. After transformation, 100 clones of each isolate were screened by PCR using ORF2 primers. Then, the ORF2 PCR products obtained from these 100 independent clones were digested with *Fnu4HI*, yielding the RFLP profiles.

For each of the 12 isolates, to investigate the diversity of RFLP profiles, PCR products of three clones characterized by the most common RFLP profiles and all clones with the less common RFLP profiles were purified with the Wizard miniprep kit (Promega), and sequenced twice. Sequencing was performed on both strands using PCR or M13 consensus primers and the DTCS sequencing kit. DNA sequences were aligned by using the CLUSTAL X software. Comparison of nucleotide

Table 1. Clinical features of the HEV epidemic (Tanefdour, Algeria, 1986–1987)

Gender	Total population (n)	Jaundiced patients [n (%)]	Fulminant cases (n)	Deaths (n)	Case fatality rate (%)
Men	1625	121 (7.4)	1	1	(0.8)
Women	1484	126 (8.4)	3	2	(1.6)

sequences generated a consensus sequence for each profile, resulting in the variants named Tanef 86/87 A to Tanef 86/87 K. Evolutionary distances were determined by the Kimura two-parameter method using the DNADIST program from the PHYLIP 3.5 package. Evidence of the phylogenetic grouping was assessed by the bootstrap method (1000 replicates). Phylogenetic trees were created with the Macintosh version of the TREEVIEW program. The full-length genome sequences used as references in this study are listed in Fig. 3.

The extent of sequence variation was determined by pairwise comparison of nucleotide sequences using the MEGA 2.1 software.

RESULTS

HEV genome amplification

HEV RNA could be amplified from 23 of 69 serum samples tested. The partial replicase and capsid genes could be amplified for all samples except one, for which ORF1 amplification failed. This cluster of HEV isolates is referred to as Tanefdour 86/87 hereafter. All PCR-positive patients had an anti-HEV response with an OD/CO ratio over 4.

Molecular characterization of Tanefdour 86/87 HEV isolates

Restriction endonuclease analysis of the 23 ORF2 PCR products revealed a *KpnI* site at nt 164 in genotype 1 strains (Burma strain). An additional *KpnI* site at position 147 was demonstrated in Tanefdour 86/87 HEV strain but was not present in the Burmese strain and in the closely related Algerian isolate named Mostaganem-80. Unlike the Burmese strain, neither the Tanefdour 86/87 HEV isolates nor Mostaganem-80 were digested by *BsmI*. *SmaI* at the specific site nt 397 in genotype 2 digested no ORF1 products of Tanefdour 86/87 HEV isolates.

To assess inter-patient genetic diversity, RFLP analysis was performed on the 448 bp ORF2 PCR products. Treatment with *Fnu4HI* revealed a large number of RFLP profiles

among the Tanefdour 86/87 epidemic strain (Fig. 1). The 23 isolates could be segregated into three RFLP profiles, named from A to C. The most common profile 'A' consisted of three bands of 120, 137 and 237 bp and grouped 18 isolates. The profile 'B' with four bands (69, 120, 137 and 187 bp) and the profile 'C' with five bands (78, 85, 110, 225 and 237 bp) gathered three and two isolates, respectively. Comparison of the nucleotide sequences of the ORF2 products from 23 HEV isolates confirmed that the Tanefdour 86/87 epidemic strain belonged to genotype 1. The ORF2 sequences from 18 of 23 isolates exhibited 99.7 to 100 % identity; those of the other five isolates exhibited 98.7 to 99 % identity.

Intra-patient heterogeneity

Intra-patient sequence diversity was studied from 12 isolates (seven randomly selected isolates displaying the main RFLP A profile, three isolates with the B profile and two isolates with the C profile). RFLP analysis of the 100 clones of these 12 isolates revealed additional heterogeneity for nine of them, named RFLP profiles D to K (Table 2). For each isolate, at least 95 % of the tested clones displayed the same RFLP profile as those obtained from the serum samples. Divergent clones only represented 5 % of the global virus population. Greater genomic diversity was observed within RFLP profile A than with RFLP profiles B and C. The most divergent isolate was AL26. Five additional RFLP profiles were characterized with RFLP A profile for this isolate (Fig. 2). Analysis of isolates AL19 (RFLP B profile) and AL01 (RFLP C profile) revealed the presence of one RFLP A clone for each isolate. To assess sequence diversity for these isolates, the nucleotide sequences of three clones exhibiting the three major RFLP profiles (A, B and C) and all the clones exhibiting the minor RFLP profiles were compared. Pairwise comparison of the 448 bp ORF2 PCR products showed that the Tanefdour 86/87 variants (Tanef 86/87 A to Tanef 86/87 K) differed by 0.54–1.2 % (mean = 0.83 %) (Fig. 3). The sequences have been deposited in

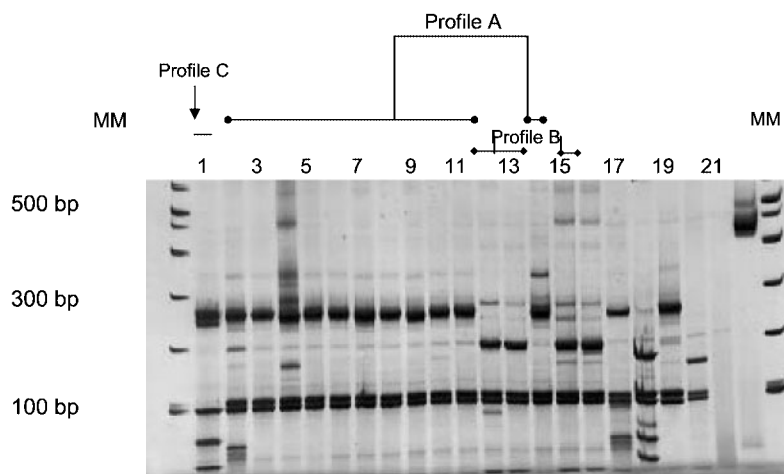


Fig. 1. Inter-patient heterogeneity of Tanefdour 86/87 isolates. Multiple RFLP profiles generated by *Fnu4HI* digestion obtained from ORF2 PCR products of Tanefdour 86/87 strains (lanes 1–15) and geographically unrelated strains [lanes 16, 17 and 19, Djibouti (genotype 1, personal data); lane 18, Nigeria (genotype 2; GenBank AF172999); lane 20, India (genotype 1, personal data); lane 21, negative control; MM, molecular mass marker]. RFLP profile type is indicated above lanes. RFLP profile A with band sizes at 120, 137, 237 bp is shared by lanes 2–11 and 14; RFLP profile B with band sizes at 69, 120, 137 and 187 bp is observed in lanes 12, 13 and 15. RFLP profile C with band sizes at 78, 85, 110, 225 and 237 bp is seen in lane 1.

Table 2. Intra-patient heterogeneity of Tanefdour 86/87 HEV isolates. RFLP analysis on 448 bp ORF2 PCR products

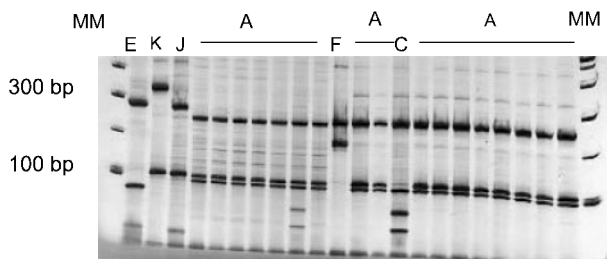
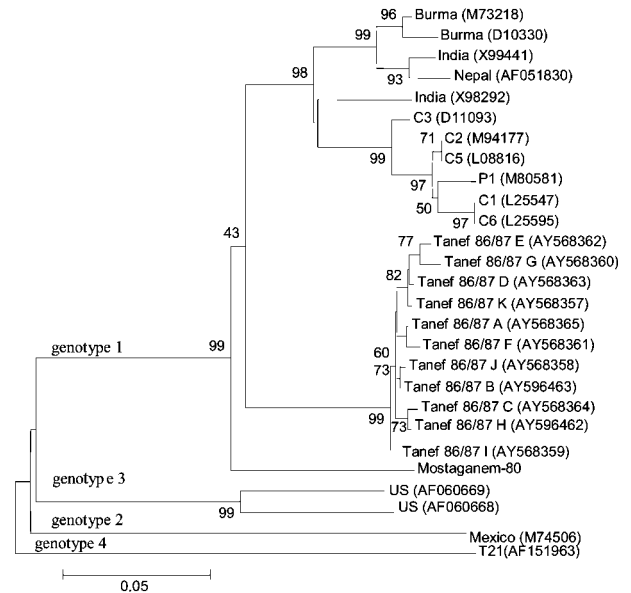
For each patient, 100 clones were tested. The numbers under RFLP profiles indicate the total number of clones matching each profile.

Isolate	RFLP profiles										
	A	B	C	D	E	F	G	H	I	J	K
AL01	1	–	99	–	–	–	–	–	–	–	–
AL04	95	2	–	2	1	–	–	–	–	–	–
AL06	100	–	–	–	–	–	–	–	–	–	–
AL09	100	–	–	–	–	–	–	–	–	–	–
AL12	98	–	–	–	–	–	–	–	–	–	2
AL15	100	–	–	–	–	–	–	–	–	–	–
AL16	–	97	–	–	–	–	3	–	–	–	–
AL19	1	99	–	–	–	–	–	–	–	–	–
AL20	–	99	–	–	–	–	–	1	–	–	–
AL25	–	–	99	–	–	1	–	–	–	–	–
AL26	95	–	1	–	1	1	–	–	–	1	1
AL28	96	–	–	–	–	–	–	–	4	–	–

GenBank under name codes: AY568365 (Tanef 86/87 A), AY596463 (Tanef 86/87 B), AY568364 (Tanef 86/87 C), AY568363 (Tanef 86/87 D), AY568362 (Tanef 86/87 E), AY568361 (Tanef 86/87 F), AY568360 (Tanef 86/87 G), AY596462 (Tanef 86/87 H), AY568359 (Tanef 86/87 I), AY568358 (Tanef 86/87 J) and AY568357 (Tanef 86/87 K).

The extent of sequence diversity in each patient was not constant within the 12 virus subpopulations studied. Three isolates (AL06, AL09 and AL15) seemed to be highly conserved with a mean diversity of 0.08%. For the nine other strains, p-distances ranged from 0.11 to 3.4% (mean = 1.02%).

Nucleotide substitutions in the Tanef 86/87 A to Tanef 86/87 K variants resulted in substantial amino acid diversity. The amino acid sequences deduced from the nucleotide sequences revealed an alanine to valine substitution at

**Fig. 2.** Intra-patient polymorphism of Tanefdour 86/87 strain, characterized from AL26, displaying the most intra-patient diversity among the 12 patients tested. A, C, E, F, J and K indicate the six different RFLP profiles observed with the AL26 isolate. MM, Molecular mass marker.**Fig. 3.** Phylogenetic analysis of the 3' end of ORF2 amplified products (448 bp) of Tanefdour 86/87 HEV variants designated Tanef 86/87 A to Tanef 86/87 K. GenBank accession numbers of reference strains are indicated beside the strain name. Genotypic designation is represented according to the Schlauder & Mushahwar (2001) scheme.

codon 585 of the capsid gene in Tanef 86/87 D, J and K variants because of a single base transition of a cytosine by a thymidine. For the Tanef 86/87 H variant, the replacement of a thymidine by a cytosine resulted in the replacement of a tyrosine by a histidine at codon 562 (Fig. 4).

DISCUSSION

HEV epidemics are mainly caused by a common source of contamination, usually drinking water resources, rather than by person-to-person transmission. Therefore, the diffusion of HEV among humans is assumed to be clonal according to a 'one outbreak, one strain' scheme. Analysis of the sequences of a limited number of HEV isolates from HEV epidemics in India supported this concept (Arankalle *et al.*, 1999; Aggarwal *et al.*, 1999).

An epidemiological survey of the jaundice epidemic that occurred in 1986–87 in the Algerian village of Tanefdour clearly demonstrated that springs were the source of HEV infections (Siriwardana *et al.*, 2003). Therefore, any of the HEV isolates from the 23 PCR-positive patients belonging to genotype 1 could be considered as a representative strain, the so-called Tanefdour 86/87 strain. Nevertheless, a slight inter-patient heterogeneity was revealed by RFLP, which divided the 23 isolates into three separate profiles (A, B and C). Moreover, RFLP analysis of 100 clones from each of 12 patients revealed intra-patient heterogeneity, with eight additional profiles (D to K) accounting for 0–5% of the clones from each patient.



Fig. 4. Amino acid alignment of partial ORF2 sequences of Tanefdour 86/87 variants. Bold characters indicate amino acid substitutions. Amino acid substitutions associated with a modification of RFLP profiles are underlined in the Burmese reference strain (M73218). The positions of amino acid residues shown on top are based on ORF2 sequence of the Burmese strain.

Nevertheless, the range of inter-patient and intra-patient nucleotide identity (96.7–99.9%) reveals overall sequence homogeneity, suggesting that a unique HEV strain, comprising multiple closely related variants, spread in the Tanefdour population.

No previous studies on HEV genome heterogeneity have included such a large cluster of HEV isolates, collected from one epidemic within an 8-week period. The inter- and intra-patient genetic diversity revealed by molecular analysis of ORF2, with major and minor variants, is consistent with a quasispecies structure of HEV epidemic strains according to the criteria for RNA viruses (Domingo *et al.*, 1985; Martell *et al.*, 1992). As a consequence of the high genetic plasticity of RNA viruses due to the lack of proofreading activity of their polymerase, the quasispecies structure describes an equilibrium status between variants in a replicating virus population. This diversity confers an advantage for survival and evolution as documented for human, animal and plant viruses (Domingo *et al.*, 1998; Schneider & Roossinck, 2001). In two extensively studied human models (acquired immunodeficiency syndrome and hepatitis C), the quasispecies diversity is generally correlated with an apparent slow or fast progression of the disease and appears to be a significant pathogenicity factor. The genomic variability of RNA viruses involved in self-limiting acute infections is currently being explored in situations other than chronic virus infections. Defective viruses with a quasispecies nature have been underlined for dengue virus type 3 from six patients in Taiwan (Wang *et al.*, 2002). Likewise, the hepatitis A virus appears to replicate as a complex distribution of mutants despite displaying conserved antigenic specificity (Sanchez *et al.*, 2003).

To date, no similar studies have been carried out on HEV. Our data, based on a large cluster of isolates from the Tanefdour epidemic, seems to confirm the quasispecies nature of HEV. Minor variants from serum samples represented no more than 5% of the overall virus population. It is difficult to detect these mutants by direct sequencing of PCR products or by studying a

few randomly selected clones. Sequence variability is not uniformly distributed in the HEV genome. Some regions within ORF1, such as the hypervariable region, are highly polymorphic whereas the amino- and carboxy-terminal ends of the capsid gene are well conserved. Antigen mapping identified immunoreactive domains in ORF2, with a major neutralization epitope located between aa 578 and 607 (Khudyakov *et al.*, 1999; Meng *et al.*, 2001; Schofield *et al.*, 2000). HEV strains from different origins or genotypes have been cross-neutralized with antibodies spanning a 166 aa epitope (Meng *et al.*, 2001). This region is located in the 3' end of ORF2 studied here. In some variants, such as Tanef 86/87 D, a single mutation modified a restriction enzyme site resulting in an amino acid substitution. In other variants, the amino acid substitutions could only be deduced from sequence data. Therefore, despite the strong immune response elicited after HEV infection, some minor virus variants within ORF2 epitopes may escape neutralization. In this study, it is clear that high serum levels of anti-HEV antibodies were associated with the persistence of HEV viraemia at the time of sampling.

Besides genetic diversity referred to as population cloud size, the quasispecies is characterized by a dynamic evolution under selective pressures such as immunological response (Schneider & Roossinck, 2001). As patients were sampled within a short period of time during the acute stage of HEV infection, it is unlikely that intra-host selective pressure led to the emergence of a major variant in each patient. The nature of the selective pressure remains to be determined. In some adults and the elderly, immune memory from past exposure to HEV could have been reactivated by Tanefdour 86/87 strain, resulting in the selection of clones less sensitive to the neutralizing effect of long-term anti-HEV antibodies.

Given the strong predominance of three RFLP profiles, especially profile A, differences in fitness between HEV variants should be considered to be a result of gradual evolution of virus subpopulations. Unfortunately, sequential samples were not collected from patients during the course of the Tanefdour epidemic.

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