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# Evidence of intratypic recombination in natural populations of hepatitis C virus

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Hepatitis C virus (HCV) has high genomic variability and, since its discovery, at least six different types and an increasing number of subtypes have been reported. Genotype 1 is the most prevalent genotype found in South America. In the present study, three different genomic regions (5′UTR, core and NS5B) of four HCV strains isolated from Peruvian patients were sequenced in order to investigate the congruence of HCV genotyping for these three genomic regions. Phylogenetic analysis using 5′UTR–core sequences found strain PE22 to be related to subtype 1b. However, the same analysis using the NS5B region found it to be related to subtype 1a. To test the possibility of genetic recombination, phylogenetic studies were carried out, revealing that a crossover event had taken place in the NS5B protein. We discuss the consequences of this observation on HCV genotype classification, laboratory diagnosis and treatment of HCV infection.

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## INTRODUCTION

Hepatitis C virus (HCV) is the major causative agent of post-transfusion hepatitis and parenterally transmitted, sporadic non-A, non-B hepatitis throughout the world (Alter & Seeff, 2000). HCV is an enveloped RNA virus classified in the family *Flaviviridae*. HCV has high genomic variability and at least six different genotypes and an increasing number of subtypes have been reported (Simmonds, 1999).

RNA viruses exploit all known mechanisms of genetic variation to ensure their survival (Domingo & Holland, 1997). Their high rate of mutation and replication allow them to move through sequence space at a pace that often makes their DNA-based host's evolution look glacial in comparison (Worobey & Holmes, 1999). Over the last two decades it has become increasingly clear that many RNA viruses add the capacity to exchange genetic material with one another. Thus, in addition to producing large amounts of the raw material of evolution (mutations), these viruses also possess mechanisms (recombination) that, in principle, allow them both to purge their genomes of accumulated deleterious changes (Muller, 1964) and to create or spread beneficial combinations of mutations in an efficient manner.

Until 1999, there was no evidence for recombination in flaviviruses, although the possibility had been considered (Blok *et al.*, 1992; Kuno, 1997; Monath, 1994). Accordingly, the vast majority of work on flaviviruses, including vaccine studies and phylogenetic analyses in which genotypes were identified and sometimes correlated with disease severity (Chen *et al.*, 1990; Leitmeyer *et al.*, 1999; Rico-Hesse, 1990), has rested on the implicit assumption that evolution in the family *Flaviviridae* is clonal, with diversity generated through the accumulation of mutational changes.

Recent studies have shown this assumption to be invalid, as homologous recombination has now been demonstrated in pestiviruses (bovine viral diarrhoea virus) (Becher *et al.*, 2001), flaviviruses (all four serotypes of dengue virus)

The GenBank/EMBL accession numbers of the sequences reported in this work are AJ438618, AJ438622 and AJ582126–AJ582135

(Holmes *et al.*, 1999; Tolou *et al.*, 2001; Uzcategui *et al.*, 2001; Worobey & Holmes, 1999), hepaciviruses (GB virus C/hepatitis G virus) (Worobey & Holmes, 2001) and Japanese encephalitis or St Louis encephalitis virus (Twiddy & Holmes, 2003). There have been few reports on recombination between HCV strains of different genotypes (Kalinina *et al.*, 2002; Yun *et al.*, 1996) and it has been suggested that these events are rare *in vivo* and that the resultant recombinants are usually not viable (Simmonds *et al.*, 1994; Smith & Simmonds, 1997).

Selected HCV genome regions within the 5′UTR, core, E1 or NS5, which have been shown to be conserved within a given HCV genotype, are used for the classification of HCV strains (Simmonds *et al.*, 1994; Simmonds, 1999). Most methods for direct HCV genotyping include amplification of different genome regions, such as the 5′UTR, core, E1 or NS4, by PCR with type-specific primers or by restriction fragment length polymorphism analysis of PCR products (Ohno *et al.*, 1997; Okamoto *et al.*, 1993; Stuyver *et al.*, 1993, 1995). Indirect HCV genotyping may be achieved by demonstration of type-specific antibodies by ELISA (Dixit *et al.*, 1995; Simmonds *et al.*, 1993). Thus, present methods of HCV genotype identification do not take recombination into account.

Given the implications of recombination for virus evolution (Worobey & Holmes, 1999) and the development of vaccines, virus control programmes, patient management and antiviral therapies, it is clearly important to determine the extent to which recombination plays a role in HCV evolution. Recombination plays a significant role in the evolution of RNA viruses by creating genetic variation. For example, the frequent recovery of recombinant isolates of poliovirus (Georgescu *et al.*, 1994; Kew & Nottay, 1984) that result from recombination involving vaccine strains shows that recombination has the potential to produce 'escape mutants' in nature as well as in experiments. Recently, recombination has also been detected in other RNA viruses for which multivalent vaccines are in use or in trials (Holmes *et al.*, 1999; Suzuki *et al.*, 1998; Worobey *et al.*, 1999). We think the potential for recombination to produce new pathogenic hybrid strains needs to be carefully considered whenever vaccines are used or planned to control RNA viruses. Assumptions that recombination either does not happen or is unimportant in RNA viruses have a history of being proved wrong (Worobey & Holmes, 1999).

In previous studies, we subtyped 72 HCV strains isolated in South America (Colina *et al.*, 1999; Vega *et al.*, 2001; San Roman *et al.*, 2002; Cristina *et al.*, 2002) by limited sequencing of the 5′UTR region. In the present study, this work was extended to include sequencing of the core and NS5B regions in order to investigate the congruence of HCV genotype determinations among the different regions of the genome. We found congruent results in 97% of cases. However, we also found evidence for recombination between type 1 subtypes of HCV in the Peruvian population.

## METHODS

**Serum samples.** Serum samples were obtained from 20 patients with chronic hepatic disease from the Hospital Nacional Edgardo Rebagliati Martins (Lima, Peru). In each case, patients were screened using an enzyme immunoassay (Innogenetics) and a confirmatory line immunoassay test (Innogenetics), according to the manufacturer's instructions.

**RNA extraction, cDNA synthesis and amplification.** HCV RNA was extracted from 140 µl serum samples with the QIAamp viral RNA kit (Qiagen) according to the manufacturer's instructions. The extracted RNA was eluted from the columns with 50 µl RNase free water. cDNA synthesis and PCR amplification of the 5′UTR, core and NS5B regions were carried out as previously described (Chan *et al.*, 1992; Norder *et al.*, 1998). To avoid false positive results, the recommendations of Kwok & Higuchi (1989) were strictly adhered to. Amplicons were purified using the QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions.

**Sequencing.** The primers used for amplification were used for sequencing the PCR fragments. The sequencing reaction was carried out using the Big Dye DNA sequencing kit (Perkin-Elmer) on a 373 DNA sequencer apparatus (Perkin-Elmer) or by manual sequencing using the Thermo Sequenase radiolabelled terminator cycle kit (Amersham).

**Sequence analysis.** The sequences for the 5′UTR plus core and NS5B regions were aligned using the CLUSTAL W program (Thompson *et al.*, 1994). Using the MEGA program (Kumar *et al.*, 1994), phylogenetic trees were created by the neighbour-joining method applied to the distance matrix obtained under the Kimura two-parameter model (Felsenstein, 1993). As a measure of the robustness of each node, we utilized the bootstrap method (1000 pseudo-replicas).

**Recombination analysis.** Putative recombinant sequences were identified with the SimPlot program (Lole *et al.*, 1999), using concatenated (5′UTR plus core plus NS5B) sequences. This program is based on a sliding window method and constitutes a way of graphically displaying the coherence of the sequence relationships over the entire length of a set of aligned homologous sequences. The window width and the step size were set to 200 bp and 10 bp, respectively. Once the recombinant strain and strains representing possible parents were identified, the likely recombination breakpoint was determined by RAT (Holmes *et al.*, 1999). Briefly, for every possible breakpoint, the sequence alignment was divided into two independent regions for which the branch lengths of a tree of the putative recombinant and its two parent sequences were optimized. The two results (likelihoods) obtained by using the separate regions were then combined to give a likelihood score for that breakpoint position, and the breakpoint position that yielded the highest likelihood was then compared, using a likelihood ratio test, to the likelihood obtained from the same data under a model that permitted no recombination. To assess whether the recombination model gave a significantly better fit to the data than the null hypothesis of no recombination, the likelihood ratios obtained using the real data were evaluated for significance against a null distribution of likelihood ratios produced by using the Monte Carlo simulation of sequences generated without recombination. Sequences were simulated 1000 times using the maximum likelihood model parameters and sequence lengths from the real data using Seq-Gen (Rambaut & Grassly, 1997).

## RESULTS

### Phylogenetic analysis of HCV strains

In order to study the congruence of HCV genotype determinations among the different regions of the genome, 5'UTR, core and NS5B sequences from four HCV strains isolated in Peru were obtained. The 5'UTR and core sequences were aligned with those from 12 other strains representative of all six HCV types isolated elsewhere for which total sequences have been obtained. The origin of the sequences and the strains used are listed in Table 1. Once aligned, phylogenetic trees were created. As can be seen in Fig. 1(A), all HCV strains included in these studies clustered according to their genotype. Strains belonging to genotype 1 clustered together with the Peruvian isolates. Inside the main cluster of type 1 strains, two different lineages could be seen, supported by very high bootstrap values. One main line represented subtype 1a (Fig. 1A, upper part, and Table 1), while the other represented subtype 1b (Fig. 1A, middle).

The same analysis was performed using NS5B sequences. The results of these studies are shown in Fig. 1(B). As can be seen in the figure, all HCV strains included in these studies again clustered according to their genotype, and inside the main type 1 cluster, two different lineages, again supported by very high bootstrap values, were observed (Fig. 1B, top). However, strain PE22, assigned to genotype 1b in the 5'UTR plus core phylogenetic tree, was now assigned to genotype 1a in the NS5B phylogenetic analysis (Fig. 1B, top). This discrepancy between the results found with the 5'UTR plus core sequences and the NS5B sequences, supported by very high bootstrap values in both trees (Fig. 1A and B), could be explained if a recombination event had taken place between putative parental strains comparable with H77 (subtype 1a) and JK1 (subtype 1b).

**Table 1.** Origins of hepatitis C virus strains

| Genotype | Name | Geographic location | Accession no. |
|---|---|---|---|
| 1a | H77 | USA | AF009606 |
| 1 | J1 | Japan | D10749 |
| 1b | JK1 | Japan | X61596 |
| 1 | HD1 | Germany | U45476 |
| 1b | K1R2 | Japan | D50476 |
| 2 | J6 | Indonesia | D00944 |
| 2 | J8 | Indonesia | D10988 |
| 3a | NZL1 | Japan | D17763 |
| 3a | K3a | Japan | D28917 |
| 3 | V-D | Germany | X76918 |
| 4a | ED43 | Egypt | Y11604 |
| 5a | EUH | UK | Y13184 |
| 6a | euhk | Hong Kong | Y12083 |
| 1 | PE8 | Peru | AJ582126, AJ582128, AJ582131 |
| 1 | PE96 | Peru | AJ438622, AJ582129, AJ582133 |
| 1 | PE108 | Peru | AJ438618, AJ582130, AJ582135 |
| 1 | PE22 | Peru | AJ582127, AJ582131, AJ582134 |

### Recombination analysis

To gain insight into a possible recombination event, a phylogenetic profile analysis was carried out for the Peruvian strain PE22 and the putative parental-like strains H77 (subtype 1a) and JK1 (subtype 1b). The results of these studies are shown in Fig. 2. As can be seen in the figure,

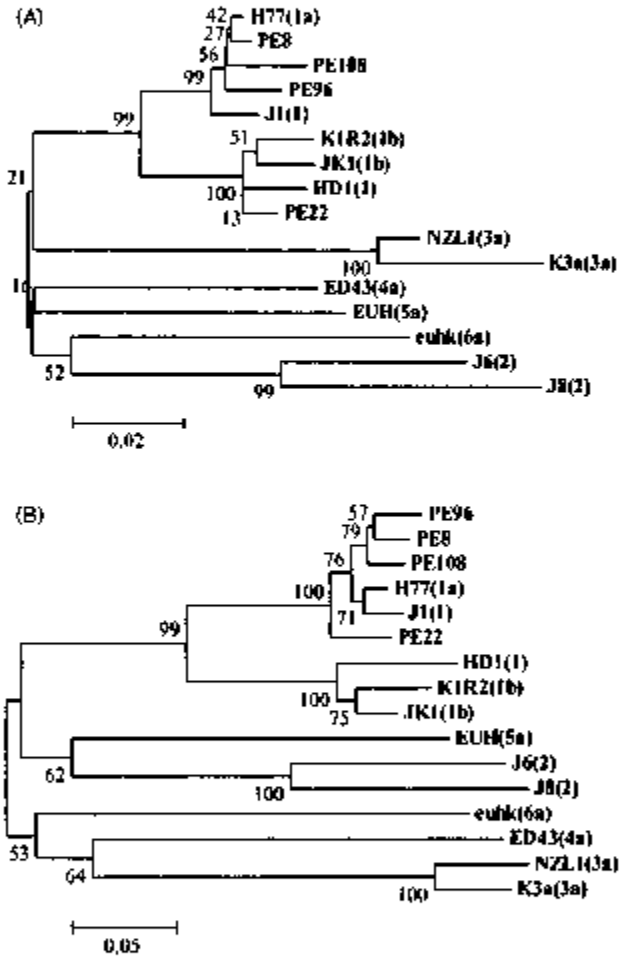


**Fig. 1.** Phylogenetic analyses of HCV strains isolated in Peru. Strains in the trees are shown by their names and their types are indicated in parentheses for strains previously described (see Table 1 for geographical location, types and accession numbers). Numbers at each node of the trees show bootstrap percentages obtained after 1000 replicates. Scale bars (number of substitutions per site) are shown at the bottom of the trees. (A) 5'UTR plus core region phylogeny. (B) NS5B region phylogeny.

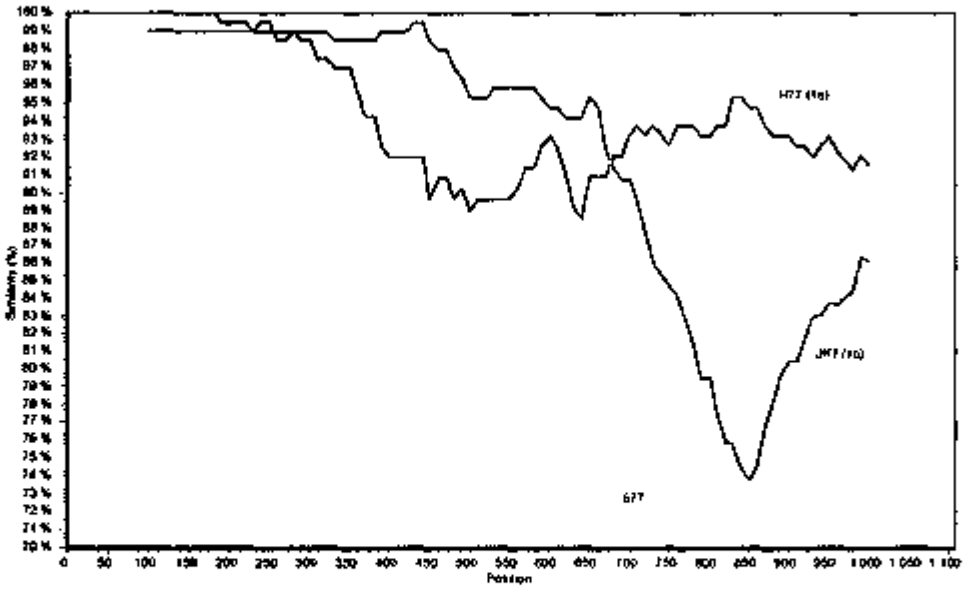

**Fig. 2.** Phylogenetic profiles of HCV sequences. The *y*-axis gives the percentage identity within a sliding window 200 bp wide centred on the position plotted, with a step size between plots of 10 bp, along the concatenated (5′UTR plus core plus NS5B) sequences. Comparison of **PE22** with H77 (subtype 1a) and JK1 (subtype 1b) is shown. The vertical line shows the recombination point (nt 677).

profile analysis of the putative parental-like (H77, JK1) and recombinant (PE22) strains showed a clearly visible point of recombination at position 677 of the analysed sequences, which corresponds to position 58 of the NS5B sequences included in this study (see Fig. 3). This position corresponds to position 8321 in the HCV genome of the putative parental strain H77.

To confirm these results, we employed the LARD method (Holmes *et al.*, 1999). Simulations of sequence evolution under the null hypothesis (i.e. no recombination) gave strong statistical support for the alternative hypothesis of recombination (Fig. 4; $P<0{\cdot}001$).

## DISCUSSION

Congruent results from genotyping HCV with different genomic regions have been repeatedly reported in the past and it has commonly been believed that recombination events are selected against or that they generally do not generate viable strains (Prescott *et al.*, 1997; Simmonds

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PE22        CCCGCTGCTTTGACTCCACGGTCACTGAGAATGATATCCGTGTTGAGGAGTCAATTTA
JK1 (1b)    -------T--------A-----------G-------------------------
H77 (1a)    -------T----------A----------GC--C------ACG------G-------

PE22        CCAATGTTGTGACCTGGACCCCCAAGCCCGCGTGGCCATCAAGTCCCTCACTGAGAGG
JK1 (1b)    ------------T--------G-G---A-ACA--T---A-G---G-----G------
H77 (1a)    ------------------------------------------------------------
```

**Fig. 3.** Alignment of the nucleotide sequences within the NS5B region. Alignment of the PE22 recombinant strain sequences with corresponding sequences of isolates JK1 and H77. The sequences shown in the figure correspond to nt 8263–8376, relative to strain H77 (AF009606, type 1a). Nucleotide identity to PE22 is indicated by a dash. An arrow shows the recombination point.

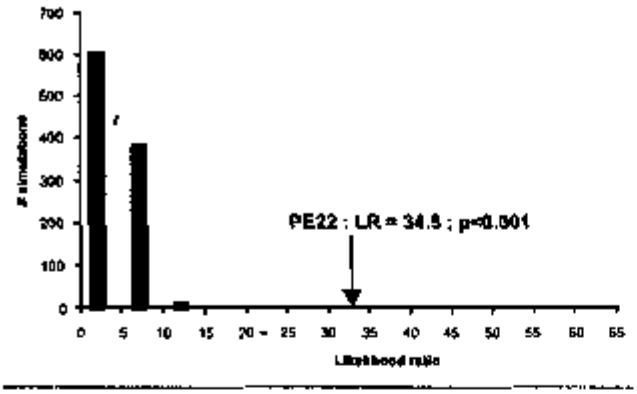


**Fig. 4.** Distribution of the likelihood ratios expected by chance. The distribution of likelihood ratios for the null hypothesis (i.e. no recombination) is shown. The *y*-axis shows the number of simulations. Likelihood ratios are shown at the bottom of the figure. The arrow shows the likelihood ratio obtained for the real dataset for the putative recombinant Peruvian strain (PE22).

*et al.*, 1994; Viazov *et al.*, 2000). However, an infectious HCV chimera comprising the complete open reading frame of a subtype 1b strain and the 5′- and 3′UTRs of a subtype 1a strain has been constructed and is infectious *in vivo* (Yagani *et al.*, 1998). Recombination in other flaviviruses has now been demonstrated on a number of occasions (Becher *et al.*, 2001; Worobey & Holmes, 2001; Worobey *et al.*, 1999; Twiddy & Holmes, 2003), and recently a natural intergenotypic recombinant (2k/1b) of HCV was identified in St Petersburg (Russia) (Kalinina *et al.*, 2002). Our phylogenetic analyses based on two different genomic regions, 5′UTR–core and NS5B, demonstrate the existence of natural intragenotypic HCV recombinant strains (1a/1b) circulating in the Peruvian population. The recombination breakpoint for non-segmented positive-strand RNA viruses, such as polioviruses and other picornaviruses (Santti *et al.*, 1999; Guillot *et al.*, 2000; Kew *et al.*, 2002), as well as members of the family *Flaviviridae*, are often located in the part of the genome encoding the non-structural proteins but sometimes in genes encoding structural proteins (Costa-Mattioli *et al.*, 2003; Martin *et al.*, 2002). Moreover, several possible recombination breakpoints have been identified in other RNA viruses, such as human immunodeficiency virus (HIV), and many more are being reported (Onafuwa *et al.*, 2003; Vidal *et al.*, 2003; Strimmer *et al.*, 2003; Najera *et al.*, 2002). The recombination point in our recombinant strain was situated in the NS5B region (see Figs 2 and 3). In the current HCV classification system, HCV strains are divided into genotypes, subtypes and quasispecies, but recombination has not yet been considered in this classification. By analogy with the nomenclature for HIV, we suggest that an HCV recombinant strain be designated a 'recombinant form' (RF), as also suggested by Kalinina *et al.* (2002). RF strains with the same number are progeny resulting from the same recombination event and thus share an identical mosaic structure; for example, the intertypic recombinants among subtypes 2k and 1b observed by Kalinina *et al.* (2002) were described as RF1_2k/1b. Accordingly, we suggest the designation RF2_1a/1b for the Peruvian strain PE22 described herein.

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