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Evidence of recombination in the capsid-coding region of type A foot-and-mouth disease virus

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Recombination is one of the factors that contribute to genetic diversity in foot-and-mouth disease virus (FMDV). Similarity and bootscan analyses have provided evidence of recombination in the capsid-coding (P1) region of the virus. In the present study, of the 14 subtype A22 field isolates that were distributed in three previously described genotypes (IV, VI and VII) based on the 1D (VP1-encoding) gene sequence (Tosh *et al*., 2002), one isolate (IND 170/88) was found to be a hybrid of genotypes VI and VII in the P1 region. VP1, VP4, the 5′ region of VP2 and the 3′ region of VP3 of this virus were characteristic of genotype VI, whereas the remaining 3′ region of VP2 and the 5′ region of VP3 were characteristic of genotype VII. No insertion or deletion was observed in the recombinant virus. Recombination in the P1 region may provide an escape mechanism for the virus.

Foot-and-mouth disease virus (FMDV) is a member of the genus *Aphthovirus* in the family *Picornaviridae* (Rueckert, 1996). The virus possesses extensive antigenic and genetic diversity, which is a major obstacle in controlling the disease (Pereira, 1977; Domingo *et al*., 1980, 1990). FMDV, like other RNA viruses, exists as closely related but non-identical genomes, termed viral quasispecies (Domingo *et al*., 1985, 1992). The extreme genetic heterogeneity is largely due to the absence of a proofreading mechanism during virus replication. From the quasispecies pool of genomes, variant populations are selected, either in the presence (Borrego *et al*., 1993) or absence of immune pressure (Domingo *et al*., 1993; Holguin *et al*., 1997). Genetic variants also arise during persistent infection of animals (Gebauer *et al*., 1988; Salt, 1993; Malirat *et al*., 1994; Woodbury, 1995) or during propagation in cell culture (Sobrino *et al*., 1983; Bolwell *et al*., 1989a). In addition to the above mechanisms, genetic diversification in FMDV can be attributed to recombination events (Pringle, 1965; McCahon & Slade, 1981; King *et al*., 1982b, 1985; McCahon *et al*., 1985; Giraudo *et al*., 1987; King, 1988; Wilson *et al*., 1988; Krebs & Marquardt, 1993). In poliovirus, homologous recombination occurs due to a copy choice mechanism (Kirkegaard & Baltimore, 1986), which may also apply to FMDV as it has a similar genome structure. Recombination has been shown to occur primarily in the 3′ half of the genome encoding the non-structural proteins, and has not been demonstrated in the capsid-coding genes of FMDV (King *et al*., 1985; King, 1988). Another report (Wilson *et al*., 1988) demonstrated that the recombination events increased much more steeply with increasing secondary structure in the regions encoding non-structural proteins compared with the structural protein-coding regions of the genome.

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FMD remains a major animal health concern in India, and the disease situation is aggravated by the presence of large populations of susceptible animals, low vaccine coverage, prevalence of multiple serotypes (O, Asia 1 and A) and unrestricted movement of susceptible animals in the country. Added to this, there is endemic cocirculation of multiple genotypes of type A virus (Tosh *et al*., 2002), and this may lead to recombination, which readily occurs between closely related strains (intratypic recombination) if multiple genotypes co-infect the host. In a preliminary antigenic analysis of type A FMDV, one-way antigenic relationships (r values; Ouldridge *et al*., 1984) revealed lower values ($r < 0{\cdot}40$) between the isolates of genotype VI and VII when tested with serum raised against one of the isolates from genotype VI (Anon., 1998–2001).

In this paper, we present the results of an analysis of the capsid-coding (P1) region of FMDV type A isolates, which provide evidence of intergenotypic recombination in one of the isolates.

Fourteen FMDV type A isolates were used in the present study; the detailed history of 13 isolates has been described previously (Nayak *et al*., 2001; Tosh *et al*., 2002). They include IND 6/92 and the type A vaccine strain (IND 17/77), whose partial and complete VP1-encoding (1D) gene sequences, respectively, are already available. Isolate IND 194/00 was recovered from cattle (on 13 March 2000) in a field outbreak in the state of Assam. The P1 nucleotide sequence data of the type A vaccine strain (IND 17/77) was taken from GenBank

Table 1. Oligonucleotide primers used to amplify and sequence type A FMDV

Primer	Sequence (5′ → 3′)	Gene location	Purpose	Reference
pNK61	GACATGTCCTCCTGCATCTG	2B	RT, PCR	Knowles & Samuel (1995)
pNK72	GAAGGGCCCAGGGTTGGACTC	2B	Sequencing	Knowles & Samuel (1995)
L01F	GTGCCCCAGTTTAAAAAGCTT	5′ UTR	PCR	Roberts & Belsham (1995)
CTL-A20	TCTCCAGGTCAGAGAAGTAGTACG	1D	Sequencing	Tosh *et al.* (2000)
CTL-A9	CTCAGGCGTGTCCGGCGGT	1C	Sequencing	Tosh *et al.* (2000)
DH6	TTGTTCTGAGTGTTGGTTGTGTG	1A	Sequencing	Sabarinath (2001)
DH1	AACAACTACTACATGCA	1A	Sequencing	Sabarinath (2001)

(accession no. AF204108) for comparison. Genotype designations were made on the basis of complete 1D gene sequences (Samuel & Knowles, 2001; Gurumurthy *et al.*, 2002; Tosh *et al.*, 2002). All the isolates were passaged three to five times in BHK-21 monolayer cell cultures. RNA was extracted from BHK-21 cell culture-infected supernatants using a Total RNA Isolation Kit (Qiagen), following the manufacturer's recommendations. The nucleotide sequences and the source of the primers used in the study are given in Table 1. cDNA was synthesized using the AMV RT Kit (Promega) and a reverse-sense primer (pNK61) at 48 °C, following the recommendations of the supplier. PCR amplification of the P1 region was performed using the HotstarTaq kit (Qiagen) and the primers pNK61 and L01F, essentially following the recommendations of the supplier. The following thermal conditions were used for amplification: 1 cycle of 95 °C for 15 min, 35 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 3 min, followed by 1 cycle at 72 °C for 10 min. Amplicons of the expected size (approximately 3·0 kb) were cleaned using the QIAquick gel extraction kit. Cycle sequencing was performed using the *fmol* kit (Promega) and Cy5-labelled primers (Table 1). The reactions were run on an ALF Express II automated sequencer (Amersham Pharmacia Biotech). Sequences were aligned using the CLUSTAL W algorithm (Thompson *et al.*, 1994), available in the OMEGA 2.0 package (Oxford Molecular). Phylogenetic analysis of the aligned sequence was performed using the neighbour-joining program (Kimura two-parameter method) in the PHYLIP package (Felsenstein, 1993). Robustness of the trees was evaluated by bootstrap analysis on 1000 replicate datasets. Phylogenetic trees were depicted using the TreeView program (Page, 1996).

Phylogenetic analysis on individual (1A, 1B, 1C and 1D) genes of the aligned sequence revealed that the position of the isolate IND 170/88 in the trees differed in these regions, suggesting that it could be a recombinant (data not shown). To examine the authentic relationships of the isolate IND 170/88 with any of the serotype A22 FMDV genotypes (IV, VI and VII) identified previously in India (Tosh *et al.*, 2002), we performed recombination analyses (Fig. 1) using SimPlot 2.5 software (Ray, 1999). Briefly, the program plots pairwise genetic similarities between a query sequence (here IND 170/88) and a set of reference sequences (representative isolates from different genotypes) in a moving window. Similarly, it also performs bootscanning (Salminen *et al.*, 1995), where it makes use of the programs SEQBOOT, DNADIST (Kimura two-parameter method), NEIGHBOR and CONSENSE from the PHYLIP package (Felsenstein, 1993). The bootstrap value (100 replicates) supporting the clustering of the query sequence with the reference sequences are plotted in a moving window along the alignment. Specifically, we performed similarity plot analysis (Fig. 1a) in a window size of 200 nucleotides moving in steps of 10 nucleotides along the alignment. The pairwise similarity values were plotted at the midpoint of the 200 nucleotide window. By examining the points at which the similarities between query (IND 170/88) and reference sequences (IND 6/92, representative of genotype VI, red; IND 69/01, representative of genotype VII, blue) increased or decreased, we could tentatively identify recombination breakpoints along the P1 region. For example, at nucleotide position 680 in Fig. 1(a) the IND 6/92 curve drops, and at nucleotide position 760 it crosses the IND 69/01 curve. Bearing in mind the window size of 200 nucleotides, this finding suggested that recombination crossover had occurred around nucleotide position 720. Similarly, at nucleotide position 1200, the IND 69/01 curve falls, and at nucleotide position 1280 it crosses the IND 6/92 curve, thereby suggesting an additional recombination crossover at around nucleotide 1240 in the P1 region (nucleotide 1240 corresponds to nucleotide 1250 in the P1 region as the gaps are stripped off in the Simplot analysis). Fig. 1(b) shows a bootscan analysis of 202 phylogenetic trees constructed on a window size of 200 nucleotides and moving in steps of 10 nucleotides along the alignment. The higher bootstrap values supporting the clustering of IND 170/88 with the representatives of genotype VI (IND 6/92, red) and genotype VII (IND 69/01, blue) suggested a mosaic pattern comprising genotypes VI/VII/VI of the IND 170/88 capsid-coding region. The recombination analyses, summarized in Fig. 1(a, b), suggested that the middle portion of the P1 region of isolate IND 170/88 is characteristic of the genotype VII sequence, and is flanked by the genotype VI sequence. Fig. 1(c)

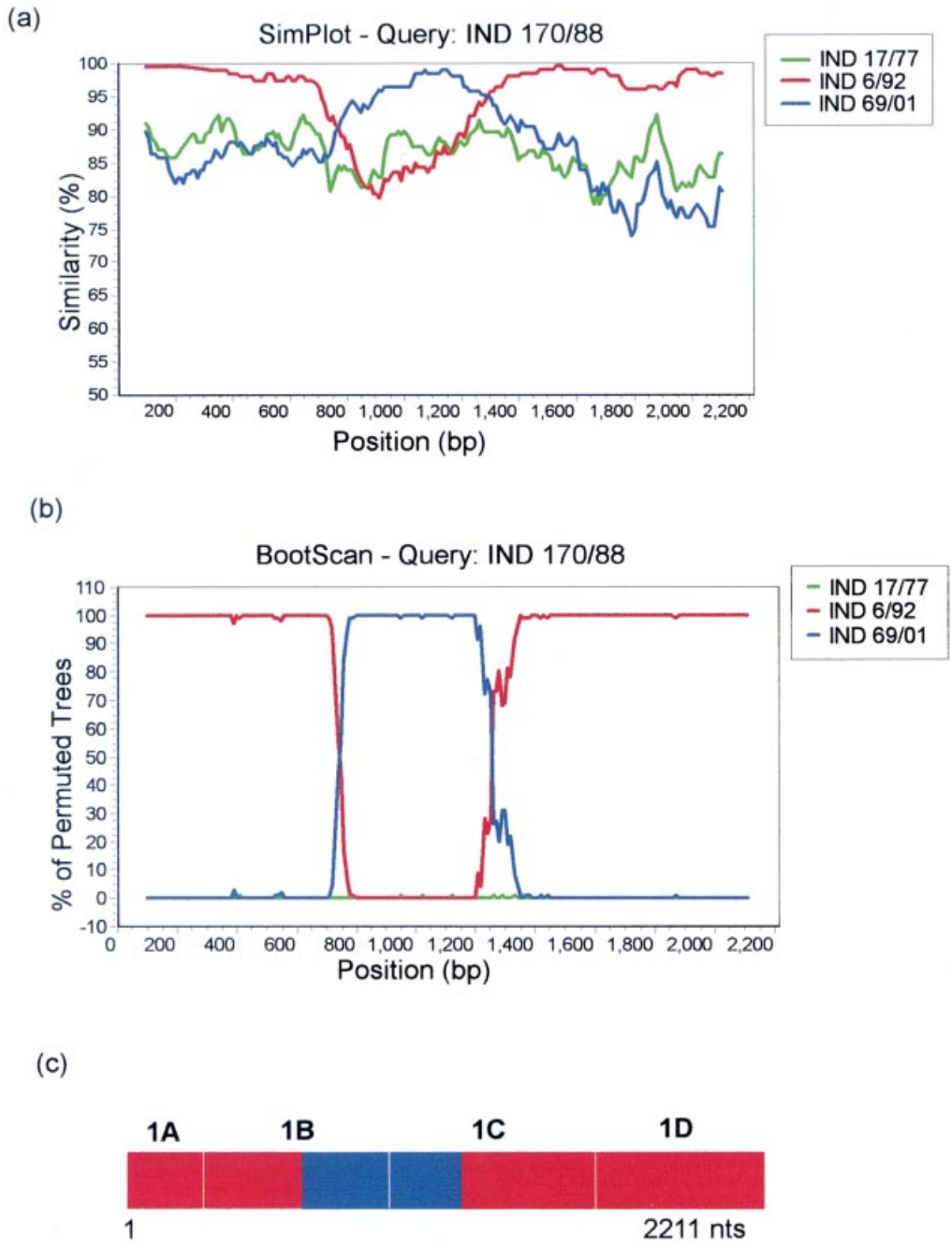

Fig. 1. Recombination analysis of the type A FMDV isolate IND 170/88, using SimPlot 2.5 software. (a) Similarity plot of IND 170/88 in comparison with representative isolates of genotype VI (IND 6/92, red), genotype VII (IND 69/01, blue) and genotype IV as an outgroup (IND 17/77, green). The *x*-axis indicates nucleotide positions along the alignment and the *y*-axis denotes the percentage similarity. (b) Bootscan plot of IND 170/88 supporting the clustering of IND 170/88 with the representative isolates as in (a). The *y*-axis indicates the percentage of bootstrap values that support the clustering of IND 170/88 with the representative isolates of different genotypes. (c) Map of the capsid-coding (P1) region of IND 170/88, showing the genotype VI fragments in red and the genotype VII fragment in blue.

provides a diagrammatic representation of the mosaic pattern in the P1 region of isolate IND 170/88, having two points of tentative recombination crossover between genotypes VI and VII. The complete VP4 and VP1, the 5′ region of VP2 and the 3′ end of VP3 are characteristic of genotype VI, whereas the remaining parts of VP2 and VP3 are characteristic of genotype VII. The two regions of crossover are in the conserved regions (or strong nucleotide sequence homology), one corresponding to VP2 βG1 and the second one to the VP3 βD sheets (Acharya *et al.*, 1989); thus, according to the proposed model for

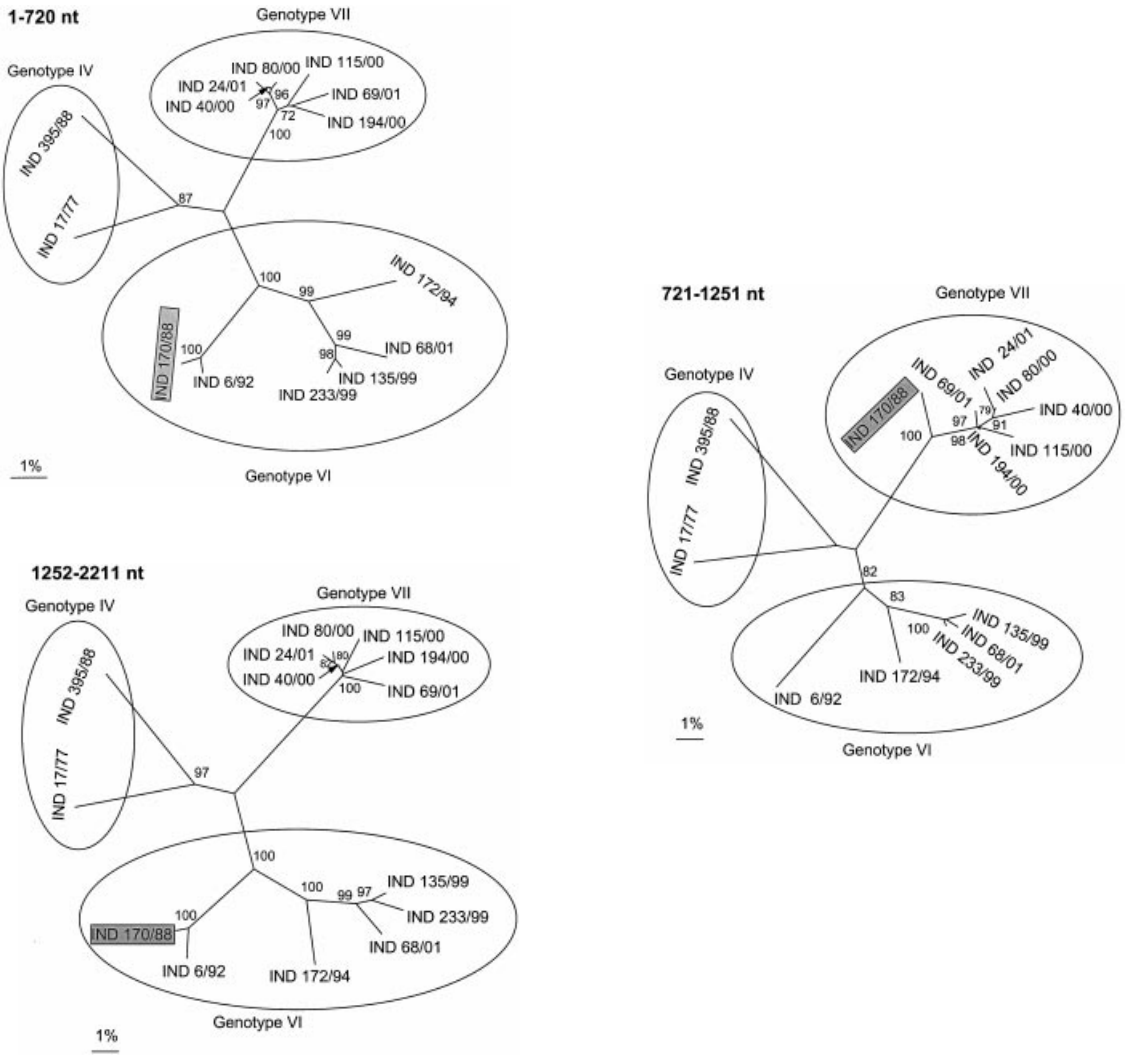

Fig. 2. Neighbour-joining trees of different P1 regions, indicating the discordant branching pattern of IND 170/88 (boxed and shaded). Nucleotide positions used in the alignments are shown above each tree; closed circles identify genotypes. Values at the nodes indicate the percentage of bootstrap values (values > 70% out of 1000 replicates are shown). Bars, 1% genetic distance.

recombination, they are the ideal places (Lai, 1992). The recombination in the P1 region may lead to antigenic diversity. As a consequence, the recombinant gets an opportunity to evade the immune response elicited by one of the parental viruses.

We have also analysed the phylogenetic informative sites (Robertson *et al.*, 1995) in the three regions tentatively identified by similarity and bootscan plots. Briefly, the analysis was performed using four sequences: the putative recombinant, a representative of each of the two genotypes apparently involved in the recombination event, and an outgroup. The number of phylogenetic informative sites supporting the grouping of the recombinant sequence (IND 170/88) with each of the other three sequences [two reference sequences, IND 6/92 (genotype VI) and IND 69/01 (genotype VII) and an outgroup, IND 17/77 (genotype IV)] were calculated. In the region 1–720 nucleotides along the alignment, the number of informative sites supporting the genotype VI origin was 35:0:0 (representing genotypes VI:VII:IV, respectively). Similarly, in the region 721–1251 nucleotides (in Fig. 1a, nucleotide position 1240 corresponds to nucleotide 1250 in the P1 region as the gaps are stripped off in Simplot analysis), the number of informative sites supporting the genotype VII origin was 1:27:2 (representing genotypes VI:VII:IV, respectively), and that in the region 1252–2211 nucleotides representing genotype VI was 60:2:1 (representing genotypes VI:VII:IV, respectively). To support further the Simplot results, phylogenetic trees were constructed for three genomic fragments corresponding to nucleotide positions 1–720, 721–1251 and 1252–2211, generated by the predicted recombination crossovers. The phylogenetic trees are presented in Fig. 2, and in all three trees the grouping of the isolate IND

170/88 with different genotypes was supported by high bootstrap values (100% out of 1000 replicates).

The findings of the Simplot and phylogenetic analyses were also supported at the amino acid level. Comparison of the deduced amino acid sequences of the first (corresponding to aa 1–240) and the last (corresponding to aa 418–737) regions of the recombinant showed fewer amino acid differences (five and nine, respectively) when compared with IND 6/92 than in comparison with IND 69/01 (13 and 33, respectively). However, the trend was reversed in the middle region, where the recombinant showed 3 and 11 amino acid differences compared with IND 69/01 and IND 6/92, respectively. The parental viruses IND 69/01 and IND 6/92 differ in 60 residues over the entire P1 region. Comparison of the amino acid residues at the antigenically critical regions showed that the recombinant virus gained residues 58, 59 and 70 of VP3 (Thomas *et al.*, 1988) from IND 69/01, and residues 175 of VP3, and 83, 138, 140, 142, 149, 154 and 170 of VP1 (Thomas *et al.*, 1988; Baxt *et al.*, 1989; Bolwell *et al.*, 1989b) from IND 6/92. From the above observation it can be suggested that the recombinant could be an antigenic hybrid, as the two parental genotypes were found to be antigenically different in one-way serological tests (Anon., 1998–2001).

The analyses identified the isolate IND 170/88 as a recombinant of genotypes VI and VII. Most of the earlier research on picornaviruses identified recombination break points in the region towards the 3′ end of the genome encoding the non-structural proteins (Cammack *et al.*, 1988; King, 1988; Wilson *et al.*, 1988; Furione *et al.*, 1993; Santti *et al.*, 1999), and only one report is available on recombination within the FMDV coat proteins involving isogenic parents (King *et al.*, 1982a). This report provides direct evidence of recombination in the structural protein-coding region in type A FMDV involving two heterogenic parents.

In conclusion, exchange of different genomic regions between viruses by recombination contributes directly to FMDV diversification and evolution. Recombination, particularly in the structural protein-coding region, as revealed in this study, may provide selective advantage to the virus, as the pre-existing immunity towards either of the parental viruses may not afford complete protection. This is of particular concern to countries where multiple FMDV genotypes co-circulate.

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