Short Communication

Evidence of rotavirus intragenic recombination between two sublineages of the same genotype

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Rotavirus G4 prevalence increased during the past decade, with one of the highest prevalences reported during rotavirus surveillance in Argentina. Intragenotype diversity analysis has led to its subdivision into lineages (I and II) and sublineages (Ia–Id). On analysis of Argentine and G4 VP7 sequences from other locations, one Argentine strain (ArgRes1723) appeared to be an intermediate between G4 sublineages Ib and Ic. Similarity and bootscanning analyses and Sawyer's test were carried out to demonstrate the recombinant nature of this strain. It was concluded that intragenic recombination occurred between sequences of sublineages Ib and Ic, with a crossover point between nucleotide positions 336 and 387. This study constitutes the first report of a mechanism of evolution in rotaviruses that is currently considered unusual – a recombination event between two strains of the same rotavirus genotype. These results will help increase current knowledge about rotavirus evolution and divergence, improving our understanding of the adaptation mechanisms used by these viruses.

Rotaviruses are the major cause of gastroenteritis in infants and young children worldwide (Estes, 2001). They are members of the *Reoviridae*, containing 11 segments of double-stranded RNA enclosed in a triple-layered capsid. The rotavirus outer layer is constituted by two proteins, VP7 (glycoprotein) and VP4 (haemagglutinin). Both molecules independently induce protective neutralizing antibodies, leading to the classification of rotavirus into G (VP7) and P (VP4) types (Estes, 2001), and are also the main targets for current vaccine-development strategies (Cunliffe *et al.*, 2002). At least 14 G types have been described so far, G1–G4 being the most common types found in humans worldwide (Desselberger *et al.*, 2001). However, from season to season the prevalent G types found in a geographical region are different, exemplified by the great increase in prevalence of G4 detected worldwide during the past decade (Gentsch *et al.*, 1996). One of the highest prevalences of G4 was reported in Argentina during national rotavirus surveillance (Bok *et al.*, 2001). Studies of intragenotype diversity led to subdivision of the genotype into several lineages and sublineages, distinctly identified by unique genomic as well as epidemiological features. The phylogenetic tree obtained from the analysis of genotype 4 VP7 gene nucleotide sequences distributed worldwide showed two major lineages, designated I and II. Lineage I was further subdivided into four sublineages, Ia–Id. Sublineages Ib and Ic were most commonly present in Argentina, and were also found to be associated with different characteristic rotavirus enterotoxins (NSP4) (K. Bok and others, unpublished data). It was noticeable that one strain, ArgRes1723, appeared to be an intermediate between sublineages Ib and Ic. Despite clustering with a high bootstrap value with sublineage Ib, it also presented the asparagine insertion (amino acid 76) characteristic of sublineage Ic (Bok *et al.*, 2002). This interesting observation could be explained by two mechanisms: intragenic recombination between strains from sublineages Ib and Ic; or sequence convergence (homoplasy) due to point mutations. Although genetic reassortment and nucleotide substitution are considered the most important mechanisms of evolution for rotaviruses (Estes, 2001; Gouvea & Brantly, 1995), Suzuki *et al.* (1998) have reported an intragenic recombination event between rotavirus strains of different serotypes. Many studies show the detection of mixed infections with two different serotypes infecting one individual, therefore increasing the probability of reassortment and/or recombination (Bok *et al.*, 2001; Gouvea & Brantly, 1995; Jain *et al.*, 2001). However, the detection of mixed infections with strains belonging to the same serotype has never been reported, as genotyping

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An alignment of the nucleotide sequences of the VP7 gene of the 43 G4 rotaviruses used in the recombination analysis is available in JGV Online.

techniques currently cannot discriminate between variants of the same genotype (Ahmed *et al.*, 1991). This study presents evidence that the ArgRes1723 strain is the evolutionary result of a genome recombination between two sublineages of the same rotavirus genotype.

Thirty Argentine VP7 gene sequences corresponding to rotavirus G4 strains were analysed, together with 13 additional sequences from other worldwide locations, collected from the GenBank database (alignments are available as a supplementary figure). The complete alignment was performed using CLUSTAL_X version 1.8, and subsequently analysed using Kimura two-parameters as a method of substitution and neighbour-joining to reconstruct the phylogenetic tree (MEGA version 2.0) (Kumar *et al.*, 2001). The statistical significance of the relationships obtained was estimated by bootstrap resampling analysis (1000 repetitions). The phylogenetic results were further confirmed by parsimony analysis using 100 bootstrap repetitions (PAUP*; Swofford, 1998). Recombination was evidenced using the

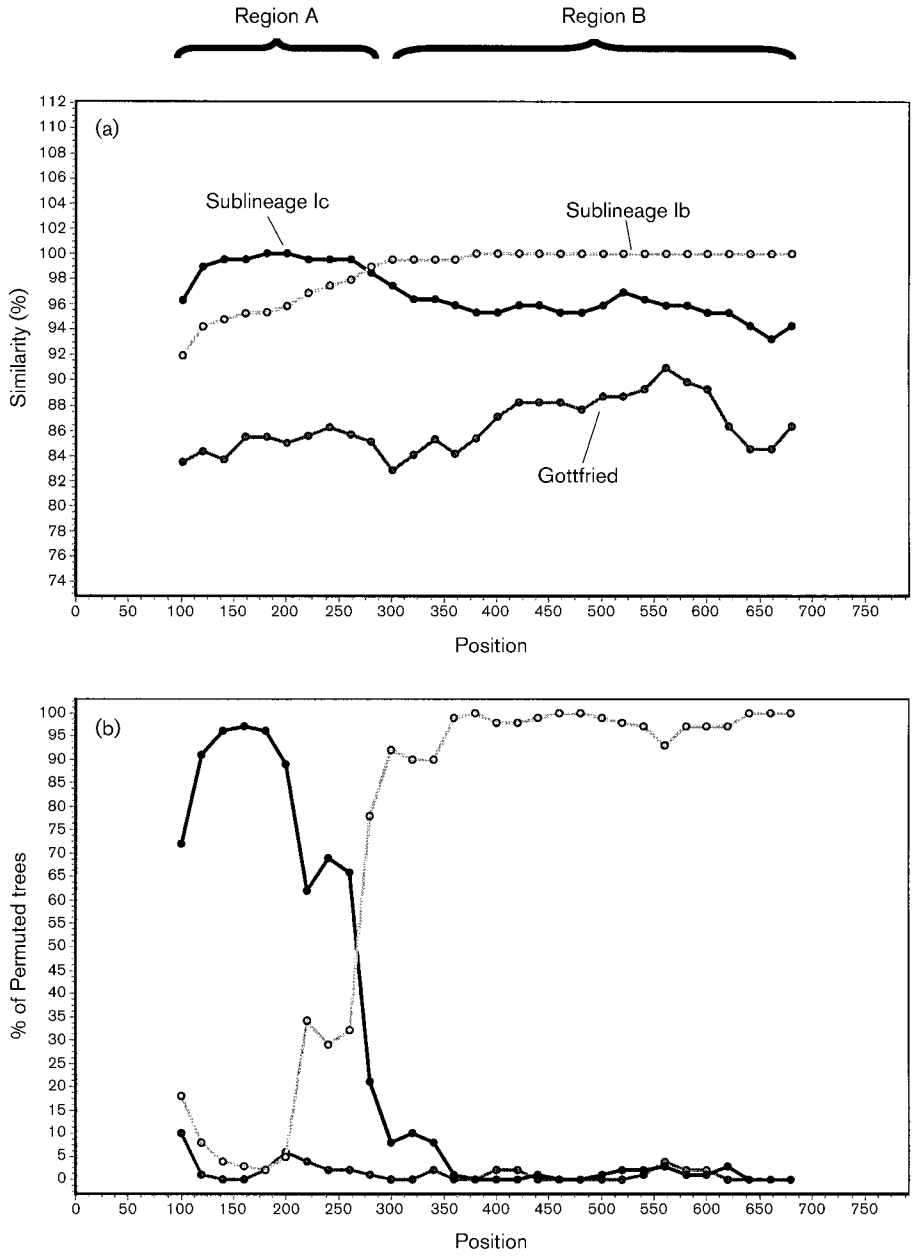

Fig. 1. (a) Similarity plots of sublineage Ic sequences, sublineage Ib and Gottfried compared to ArgRes1723. Plots were calculated using the SIMPLOT program with a sliding window size of 200 bp and a step size of 20 bp. (b) Bootscanning analysis used to resolve the recombination event with a window size of 200 bp and a step size of 20 bp (gap strip off; 100 bootstrap replicates and neighbour-joining tree analysis).

SIMPLOT program version 3.2 (Lole *et al.*, 1999), with similarity and bootscanning analysis (Salminen *et al.*, 1995) and GENECONV, a computer algorithm based on Sawyer's test (Sawyer, 1989). The putative recombinant sequence (ArgRes1723) was compared with strains from the two proposed parental sublineages (20 from sublineage Ib and 8 from sublineage Ic), and one sequence of a known outgroup (Gottfried). Informative sites were identified where two sequences shared one specific nucleotide but two additional sequences shared a different nucleotide.

The similarity plots showed that the ArgRes1723 sequence was more similar to sublineage Ic in the first portion of the sequence (named region A; Fig. 1a), then drastically diverged to become very similar to sublineage Ib in the second portion of the sequence (region B). Bootscanning analysis (Fig. 1b) showed the break point, estimated to be between nucleotides 336 and 387, after mapping the informative sites.

To support the SIMPLOT results, phylogenetic trees (Fig. 2) were constructed for each of the genomic regions: A, nucleotides 76–336; B, nucleotides 337–866 (nt positions refer to the Gottfried strain). Phylogenetic trees showed the recombinant nature of ArgRes1723, with a bootstrap value of 56 % in region A and 99 % in region B. Despite the low bootstrap value (56 %) of region A in the branch node of sublineage Ic, an internal branch that included ArgRes1723 showed a bootstrap value of 93 %.

We also analysed 17 phylogenetically informative sites obtained from four sequences: query, ArgRes1723; sublineage Ic, ArgMis864; sublineage Ib, ArgTuc737; outgroup, Gottfried. In this case three of 17 informative sites corresponded to the asparagine insertion, while most sites (11 of 17) were located at the third codon positions, leading to synonymous substitutions only. Therefore the recombination event probably did not result from sequence convergence due to positive selection, given that selective constraints operate at the amino acid level. That is, natural selection is unlikely to have produced a false signal for recombination. In addition, one informative site was located at a non-coding site, and the remaining two informative sites were located at first codon positions where no amino acid changes were detected. One amino acid change was observed in the informative site codon 465, but this was due to the difference in the first codon position compared with the Gottfried sequence. Moreover, sequence convergence could not have originated by random fluctuation of the sequences, as no informative sites were detected supporting one of the possible associations: ArgRes1723 with Gottfried. Additionally, the recombination event was strongly supported by Sawyer's test. This analysis identified significant ($P<0{\cdot}001$) common fragments, one between ArgRes1723 and ArgMis864 (152–386), and another between ArgRes1723 and ArgTuc737 (337–747; $P<0{\cdot}001$).

From these results it was concluded that intragenic recombination occurred between sequences of sublineages Ic and Ib, with a crossover point between nucleotides 336 and 387. Although possible RT-PCR artefacts could have caused the recombination between sequences in samples

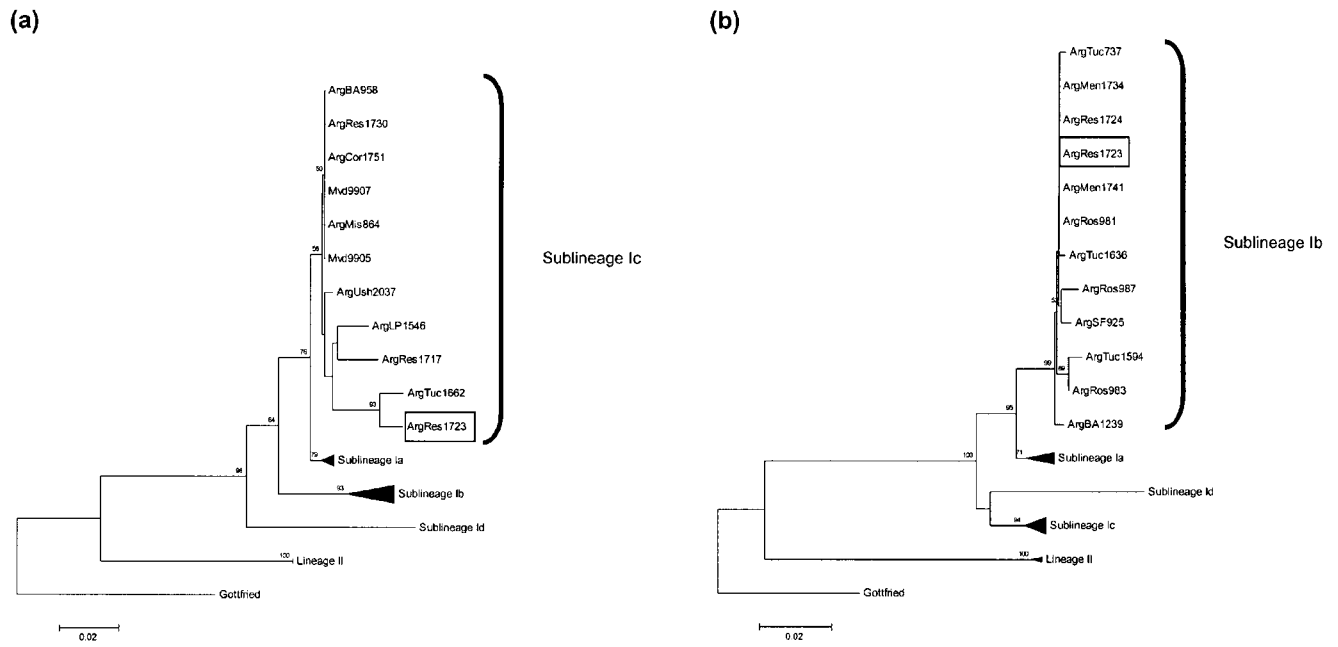

Fig. 2. Phylogenetic trees for sublineages (a) Ic and (b) Ib, corresponding to regions A and B (A, nt 76–336; B, nt 337–866), indicating the different phylogenetic positions of ArgRes1723 (boxed). Bootstrap indicated by node values (values <50 % not shown).

with mixed infections, the amplification and sequencing assays were independently repeated to minimize this possibility. Sublineage Ib and Ic strains were the most prevalent variants circulating in Argentina during 1997–98, so if recombination between rotavirus strains was possible, it would be expected to occur between these two variants. However, the generated strain might not show improved fitness, limiting its dissemination and consequent detection, which was the case throughout the remainder of the national surveillance study.

This is the first study reporting evidence of intragenic recombination between rotavirus strains of the same genotype. In addition to point mutations, there is now evidence that genetic diversity within a rotavirus genotype is also generated by recombination. Intragenic recombination might be an important mechanism used by rotaviruses to generate escape mutants avoiding the host's immune system. This observation becomes especially meaningful if the recombination process occurred between strains of a sublineage (Ib) which was already considered established in the population, and another sublineage (Ic) which was a recent introduction in Argentina (Bok *et al.*, 2002). These results raise the possibility that rotavirus recombination might not be an extraordinary event, but rather a phenomenon likely to occur frequently but to be detected rarely. The information presented here increases current knowledge about rotavirus evolution and the origin of divergence, and improves our understanding of the adaptation mechanisms used by rotaviruses.

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