Molecular epidemiology of human P[8],G9 rotaviruses in Hungary between 1998 and 2001

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Increasing numbers of studies have documented the widespread distribution of human G9 rotaviruses and demonstrated that these strains may represent a fifth epidemiologically important G serotype. Serotype G9 strains were identified in Hungary for the first time in the 1997–1998 rotavirus season. Contrary to numerous surveys that reported several unexpected P–G combinations among recent G9 isolates (e.g. genotypes P[4], P[6] and P[19]), all Hungarian strains characterized to date possess the globally most common P-type, P[8], which was found among the first G9 isolates that were identified during the 1980s in the USA (WI61) and Japan (F45). To study the genetic variability within Hungarian G9 strains, RNA profile analysis and nucleotide sequencing were performed on a subset of samples that were collected between 1998 and 2001. These strains could be classified into four major RNA profiles, of which two were characteristic for epidemiologically major and two for epidemiologically minor G9 strains. Phylogenetic analysis demonstrated substantial sequence differences between the VP7 gene of Hungarian G9 strains and early strains that were isolated in the USA (WI61), Japan (F45) and India (116E) and a few recently identified isolates, e.g. from China (97’SZ37) and the USA (OM67) (<90 % nucleotide sequence similarity). In contrast, the VP7 genes of Hungarian G9 strains were related very closely to the vast majority of G9 strains that were isolated in a variety of countries over the last several years (>96 % nucleotide sequence similarity). With respect to the VP4 gene, Hungarian G9 rotaviruses fell into two of the major genetic lineages of genotype P[8], one corresponding to the epidemic strains (lineage II; P-like) and the other for two unique strains (lineage I; Wa-like), suggesting independent introduction of distinct P[8],G9 strains into Hungary or genetic reassortment between locally circulating P[8] strains and descendants of G9 isolates that were imported into the country at an earlier time. The unexpected heterogeneity found for G9 VP7 genes from several countries suggests that genetic variation among these strains has not yet been fully explored.

INTRODUCTION

Rotavirus is the major aetiological agent of severe gastroenteritis in infants and young children. There are an estimated 400 000–800 000 fatal rotavirus cases each year, mostly in developing countries. In addition, despite improvements in living conditions and sanitation in developed countries, the annual incidence of rotavirus infections remains high and costs of treatment are significant. This magnitude of mortality and economic burden justify the need for introduction of effective control measures. As traditional public health interventions are inefficient in the control of rotavirus infections, the most promising alternative may be a mass vaccination programme (Kapikian & Chanock, 1996; Bresee et al., 1999; Glass et al., 1999).

Rotaviruses, which are members of the family Reoviridae, possess a genome of 11 dsRNA segments that are enclosed in a triple-layered capsid. Rotaviruses are classified into sero-
groups, designated A to G. Of these, group A rotaviruses are the major enteropathogenic viruses in infancy (Kapikian & Chanock, 1996). Based on antigenic differences of the surface antigens, VP7 and VP4, group A rotaviruses can be further classified into G and P serotypes, respectively. Nucleic acid-based genotyping methods (e.g. sequencing, RT-PCR or probe hybridization) to identify the genetically distinct genes that specify individual G and P serotypes have also been developed (Flores et al., 1989; Gouveia et al., 1990; Larralde & Flores, 1990; Gentsch et al., 1992).

In epidemiological surveys, both antigenic typing with serotype-specific mAbs and genotyping techniques are used frequently for determination of G serotype specificity. In contrast, as mAbs to common P serotypes are cross-reactive and neutralization assays are cumbersome and require tissue culture-adapted strains, genotyping methods are used widely for P type identification.

To date, 10 G serotypes (G1–G6, G8–G10 and G12) and 11 P genotypes (P[1], P[3]–P[6], P[8]–P[11], P[14] and P[19]) are known to cause infection in humans (Nakagomi et al., 1994; Hoshino & Kapikian, 2000; Jagannath et al., 2000; Okada et al., 2000). Although the theoretical number of P–G antigen combinations that could be generated from individual types is very large, only a few of these have epidemiological significance. Historically, four major strains (P[8],G1; P[4],G2; P[6],G9; and P[8],G4) have been linked to about 90–95% of hospital-associated infections (Gentsch et al., 1996). The G serotype specificities of these four strains are the targets of vaccine development, as exemplified by the first licensed rotavirus vaccine, called rehsus rotavirus (RRV) tetravalent vaccine (RRV-TV) or RotaShield, the trade name assigned by its US manufacturer. This vaccine contained the RRV parent strain, which shares VP7 specificity with that of human G3 strains, and three rehsus–human mono-reassortant strains, with the VP7 genes of human rotavirus serotypes G1, G2 and G4, respectively, and the remaining 10 genes of the RRV parent (Midthun et al., 1985). RotaShield was approved in 1998 by the Food and Drug Administration for routine administration in US children. In 1999, however, due to a rare association between vaccine use and severe bowel obstruction in vaccinees, the vaccine was withdrawn from the US market (CDC, 1999a, b). Despite these serious adverse effects of vaccination with RotaShield, further efforts at finding safe and effective vaccines are under way (Clack et al., 1996; Bernstein et al., 1998; Clements-Mann et al., 2001).

In anticipation of possible national immunization programmes against rotavirus, intensified surveillance has begun worldwide to assess rotavirus disease burden in countries that are considering use of vaccines and to determine the relative importance of common and rare serotypes in individual countries. These studies will help to determine the need for rotavirus vaccines in countries worldwide and in the formulation of vaccines that contain the most common serotype antigens that are capable of eliciting adequate type-specific or heterotypic immunity.

Strain prevalence surveys, supported by improved detection and typing methods, have led to increased identification of serotypes that were previously considered to be rare or that had never been detected before. Some of these may only have local significance (e.g. G5 in parts of Brazil and G8 in Malawi; Gouveia & Santos, 1999; Bok et al., 2001b; Cunliiffe et al., 2001b), but at least one serotype, G9, was demonstrated to have dispersed worldwide (Ramachandran et al., 1996; Unicombe et al., 1999; Griffin et al., 2000; Iturriza-Gomara et al., 2000; Palombo et al., 2000; Araújo et al., 2001; Bok et al., 2001a; Cunliiffe et al., 2001a; Bányai et al., 2004).

Serotype G9, as the sixth recognized human VP7 specificity, was first identified during 1983 in the USA and from 1985 to 1989 in Japan, India, Yugoslavia and Thailand (Clack et al., 1987; Nakagomi et al., 1988, 1990; Urasawa et al., 1992; Zizdic et al., 1992; Das et al., 1993). Representative US and Japanese G9 strains carried P[8] VP4 specificity, possessed a ‘long’ electrophoretic type (E-type; also referred to as genome pattern or RNA profile) and, as determined by whole-genome RNA–RNA hybridization, exhibited close genomic relatedness to strain Wa (P1A[8],G1), the prototype of the most common human rotaviruses, designated the Wa genogroup (Nakagomi et al., 1990). The Indian G9 strains, which were only identified in neonates who excreted rotavirus without symptoms of diarrhoea, had P[11] or P[6] VP4 specificity, ‘long’ E-types and were also members of the Wa genogroup (Das et al., 1993; Laird et al., 2003). The P[11] VP4 gene is related closely to the cognate gene of bovine strain B223, suggesting that these strains were derived by reassortment with bovine rotaviruses (Gentsch et al., 1993). Two G9 strains that were detected in Thailand had P[19] genotype specificity and genomic relatedness with porcine rotaviruses (Urasawa et al., 1992; Okada et al., 2000). Although highly diverse, the small number of reports of G9 detection before 1990 suggested that these strains were a relatively uncommon cause of gastroenteritis.

Beginning in the mid-1990s, G9 strains apparently emerged globally (Ramachandran et al., 1996; Unicombe et al., 1999; Griffin et al., 2000; Widdowson et al., 2000; Cunliiffe et al., 2001a). The G9 rotaviruses identified more recently exhibited a great variety of genomic constellations, formed predominantly by reassortment of VP4 and VP7 genes into both ‘long’ and ‘short’ E-type strains. For example, P[4],G9 ‘short’ E-type strains were identified in Thailand; P[4],G9 ‘long’ E-type strains were found in Brazil; P[6],G9 ‘short’ E-type strains were deleted in the USA, India and Bangladesh; P[6],G9 ‘long’ E-type strains were detected in India, Bangladesh and Kenya; and P[8],G9 ‘long’ E-type strains circulated in the USA, Bangladesh, Libya, Cuba, Kenya, India and Japan (Unicombe et al., 1999; Griffin et al., 2000; Oka et al., 2000; Ramachandran et al., 2000; Araújo et al., 2001; Cunliiffe et al., 2001a; Jain et al., 2001; Zou et al., 2001).

During a 1997–1998 longitudinal study in Hungary, P[8],G9 ‘long’ E-type strains were detected for the first time in our country. Subsequently, during 1999–2001, P[8],G9 rotaviruses were detected at an increased prevalence and were the second most common strain detected during this period.
(Bányai et al., 2004). In this report, we describe the findings of RNA profile analysis, which we used to study geographical and temporal changes within G9 strains that circulated between 1998 and 2001 in Hungary. Moreover, sequence and phylogenetic analyses were performed for the outer capsid genes (VP7 and VP4) to investigate the genetic relationship among Hungarian strains and G9 strains identified in other countries, in order to help to understand the epidemiology and evolution of these emerging rotaviruses.

**METHODS**

**Specimens.** Serotype G9 rotaviruses characterized in this report were identified during a surveillance study by using mAb-based enzyme immunoassay serotyping (Coulson et al., 1987; Taniguchi et al., 1987) and RT-PCR (Gentsch et al., 1992; Das et al., 1994) genotyping (Bányai et al., 2004). Of 219 specimens that were found to be positive for serotype G9 rotaviruses between 1998 and 2001 (Table 1), nine strains were selected for sequence analysis on the basis of differences in RNA profiles and year and place of identification. Of these, eight samples were collected in Budapest, Hungary [Hun9 (1997–1998 rotavirus season); BP785/00, BP850/00 and BP857/00 (1999–2000); and BP626/01, BP641/01, BP744/01 and BP1829/01 (2000–2001)] and one sample was collected in Baranya County, Hungary [BC4572/00 (1999–2000)]. The two study areas are 150–200 km apart.

**RNA extraction.** Rotavirus dsRNA was extracted from diluted faecal specimens by the guanidine thiocyanate/glass milk method, as described previously (Gentsch et al., 1992).

**RNA profile analysis.** We analysed the viral genome by PAGE and silver staining (Laemmli, 1970; Dolan et al., 1985). We used the tissue culture-adapted strain WI61 (P[8],G9) as a control (Clark et al., 1987).

**Nucleotide sequencing.** The outer capsid genes VP7 and VP4* (a fragment of the VP4 gene) were reverse-transcribed and the resulting cDNA was amplified by using the consensus primers 9con1 and 9con2, and con3 and con2, respectively (Gentsch et al., 1992; Das et al., 1994). Amplicons were run in a low-melting-point agarose gel, subsequently excised and then extracted with a QIAquick Gel Extraction kit (Qiagen). PCR products were labelled and run on an automated sequencer (ABI Prism 310; Applied Biosystems) by using the individual primers that were used to generate the RT-PCR products. Where needed, primers homologous to internal regions of the RT-PCR products were utilized to complete the sequence (not shown). Dye-labelled products were extracted by sodium acetate/ethanol precipitation. The pellet was resuspended in formamide and then run on an automated sequencer (ABI Prism 310; Applied Biosystems).

**Sequence and phylogenetic analysis.** Nucleotide sequences were aligned with those available in GenBank/EMBL/DBJ by using the program DAMBE (Xia & Xie, 2001) and edited with the program GeneDoc v2.3 (Nicholas et al., 1997). The phylogenetic relationship between strains was estimated by maximum-likelihood and distance matrix analysis followed by the neighbour-joining tree reconstruction algorithm. Robustness of the trees was assessed by bootstrap analysis. The computer simulation was performed with the PHYLIP 3.5 and MEGA2 software packages (Felsenstein, 1989; Kumar et al., 2001).

**GenBank accession numbers.** Partial sequences of the VP7 and VP4 genes were deposited in GenBank under the following accession numbers: VP7, AJ605303–AJ605311; VP4, AJ605312–AJ605320. G9 VP7 and P[8] VP4 sequences that are available for public use were downloaded from GenBank/EMBL/DBJ after a sequence similarity search was done with the BLAST search algorithm (Altschul et al., 1997).

**RESULTS**

**RNA profile analysis of Hungarian G9 strains**

When Hungarian P[8],G9 strains were analysed by electropherotyping, four distinct RNA profiles were identified. When a strain representative of each RNA profile was subjected to RNA profile analysis, four distinct RNA profiles were identified. When a strain representative of each RNA profile was subjected to RNA profile analysis, four distinct RNA profiles were identified.

**Table 1. Temporal and geographical distribution of E-types associated with G9 type specificity**

Data in parentheses are no. samples for which G type was examined and G9 specificity was determined successfully.

<table>
<thead>
<tr>
<th>Season</th>
<th>Area*</th>
<th>No. rotavirus-positive samples subjected to RNA profile analysis</th>
<th>No. strains with indicated E-type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BC</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>1998–1999</td>
<td>BP</td>
<td>351</td>
<td>B: 1 (0)</td>
</tr>
<tr>
<td></td>
<td>BC</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>1999–2000</td>
<td>BP</td>
<td>604</td>
<td>C: 1 (1)</td>
</tr>
<tr>
<td></td>
<td>BC</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td>2000–2001</td>
<td>BP</td>
<td>831</td>
<td>D: 1 (1)</td>
</tr>
<tr>
<td></td>
<td>BC</td>
<td>325</td>
<td></td>
</tr>
</tbody>
</table>

*BC, Baranya County; BP, Budapest.
in different countries, phylogenetic analysis was performed to assess the evolutionary relationships among Hungarian G9 strains and compare them to G9 strains that were isolated in the USA, was included as a reference P[8],G9, 'long' E-type strain. Representative strains with the indicated RNA profiles were as follows: A, Hun9, BP850/00, BP857/00, BC4572/00 and BP641/01; B, BP785/00; C, BP626/01 and BP1744/01; D, BP1829/01. Triangles in the line drawings of E-types B, C and D (to the right of the gel presentation) indicate major differences in the genome pattern found, compared to pattern A (designated as the prototype Hungarian E-type associated with G9 VP7 specificity).

FIG. 1. RNA profiles of P[8],G9 rotavirus strains identified in Hungary between 1998 and 2001. Isolate WI61, detected in the early 1980s in the USA, was included as a reference P[8],G9, 'long' E-type strain. Representative strains with the indicated RNA profiles were as follows: A, Hun9, BP850/00, BP857/00, BC4572/00 and BP641/01; B, BP785/00; C, BP626/01 and BP1744/01; D, BP1829/01. Triangles in the line drawings of E-types B, C and D (to the right of the gel presentation) indicate major differences in the genome pattern found, compared to pattern A (designated as the prototype Hungarian E-type associated with G9 VP7 specificity).

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All Hungarian G9 strains and most recently detected strains from other countries formed a closely related cluster that was distinct from the original P[8],G9 strains that were isolated during the 1980s in the USA (WI61) and Japan (F45, AU32) (Fig. 2). Other isolates from the 1980s and a few recent strains clustered together, but separately from most recently isolated G9 strains. For example, the VP7 of P[19],G9 strains identified in Thailand in 1989 (e.g. Mc323) and in Vietnam in 1998–1999 (e.g. 684VN) formed a separate cluster, although they shared up to 97.3 % sequence similarity with some novel G9 strains, a level similar to that observed within members of the globally emerged lineage (data not shown). Another branch included G9 strains that were identified in the late 1990s and 2000 in far-eastern Asian countries (e.g. T203 in China and K-1 in Japan). A few other recent isolates (e.g. OM67 from the USA and 97’SZ37 from China) were related more distantly to members of recent lineages than to strains identified in the 1980s (Fig. 2).

Phylogenetic analysis of VP8* sequences indicated that Hungarian P[8],G9 strains clustered in two separate branches (Fig. 3). Seven of nine study strains (i.e. E-type A strains Hun9, BP850/00, BP857/00, BC4572/00 and BP641/01 and E-type C strains BP626/01 and BP1744/01) clustered together and shared >98 % sequence similarity with each other (nt 33–564; data not shown). Two of those seven strains for which a longer nucleotide sequence was determined (Hun9 and BP857/00; nt 33–850) shared highest similarity (>98 %) with other G9 strains identified recently in the UK and India (e.g. 466/97 and 02-RV35, respectively) and with G1 isolates identified in Taiwan (strain TF101) and Malawi (e.g. OP351 and OP601). All of these strains and others, including reference strain P, a P1A[8],G3 isolate from the USA (Wyatt et al., 1983), formed a genetic cluster (designated P-like) that was distinct from the three earlier described VP8* lineages (i.e. Wa-like or lineage I, F45-like or lineage II, and MW670-like or lineage III) (Maunula & von Bonsdorff, 1998; Gouvea et al., 1999). However, bootstrap analysis demonstrated that P-like strains, including Hun9 and BP857/00, are actually genetically divergent members of lineage II (F45-like; Fig. 3). This conclusion is supported by the findings of both non-silent (nucleotide positions 494, 576 and 593; corresponding amino acid positions are 162, 189 and 195, respectively) and silent nucleotide substitutions (nucleotide positions 87, 105, 126, 141, 171, 297, 588, 609, 672 and 676) that are highly conserved among members of these two lineage II genetic clusters (Table 2). In contrast, E-type B (BP785/00) and D (BP1829/01) strains were distinct from these seven Hungarian G9 strains (<91 % similarity; nt

Sequencing and phylogenetic analysis of VP7 and VP8* genes

To assess the evolutionary relationships among Hungarian G9 strains and compare them to G9 strains that were isolated in different countries, phylogenetic analysis was performed for the outer capsid proteins VP7 and VP4.

Analysis of nucleotide sequences revealed a close relationship among the G9 VP7 genes of Hungarian strains (>98 % sequence similarity; data not shown) and a similar extent of sequence similarity to most G9 strains that have been identified recently in European, American, African and Asian countries (>96 %). A unique strain, CIT-254RV (identified in Ireland), was related more closely to members of the globally emerged G9 lineage, despite having relatively low sequence similarity (95.2–93.0 %) to those strains, including the Hungarian G9 strains (Fig. 2).

Analysis of the seasonal distribution of the four E-types demonstrated that E-type A was the only strain found in the late 1990s and 2000 in far-eastern Asian countries (e.g. T203 in China and K-1 in Japan). A few other recent isolates (e.g. OM67 from the USA and 97’SZ37 from China) were related more distantly to members of recent lineages than to strains identified in the 1980s (Fig. 2).

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Fig. 1. RNA profiles of P[8],G9 rotavirus strains identified in Hungary between 1998 and 2001. Isolate WI61, detected in the early 1980s in the USA, was included as a reference P[8],G9, 'long' E-type strain. Representative strains with the indicated RNA profiles were as follows: A, Hun9, BP850/00, BP857/00, BC4572/00 and BP641/01; B, BP785/00; C, BP626/01 and BP1744/01; D, BP1829/01. Triangles in the line drawings of E-types B, C and D (to the right of the gel presentation) indicate major differences in the genome pattern found, compared to pattern A (designated as the prototype Hungarian E-type associated with G9 VP7 specificity).
Fig. 2. Phylogenetic tree of the G9 VP7 gene. The tree was obtained by neighbour-joining simulation of the nucleotide sequence for positions 49–943. Bootstrap values > 60 are indicated. For each strain, the associated P-type specificity (where available) and the country of origin are shown. An outgroup, serotype G4 (GenBank accession no. AB012079), was included to reveal a more accurate genetic relationship among G9 sequences. Bar, 0.01 phylogenetic distance.
Fig. 3. Neighbour-joining tree obtained from nucleotide sequences of the P[8] VP8* gene (nt 86–730). Bootstrap values $\geq 60$ are shown. For each strain, the associated G-type specificity and the country of origin are also shown. A genotype P[6] sequence (GenBank accession no. AJ278256) was included to serve as outgroup. Bar, 0.01 phylogenetic distance.
33–564). They shared 96.3% nucleotide sequence similarity with each other (nt 33–850) and were related more closely to reference strain Wa (lineage I) than to strains in other clusters (e.g. P-like, F45-like or MW670-like strains).

**DISCUSSION**

From its first detection in 1983 until the early 1990s, serotype G9 was rarely identified globally as a cause of diarrhoea, although it was known as a common cause of asymptomatic infections in Indian neonates (Clark et al., 1987; Nakagomi et al., 1990; Urasawa et al., 1992; Zizdic et al., 1992; Das et al., 1993). Beginning in the mid-1990s, there was a remarkable increase in the detection of G9 strains as a cause of gastroenteritis and over the last 7 years or so, G9 rotaviruses have emerged as one of the most epidemiologically important strains worldwide in both developed and developing countries (Ramachandran et al., 1996; Griffin et al., 2000; Iturriza-Gomara et al., 2000; Palombo et al., 2000; Widdowson et al., 2000; Araújo et al., 2001; Bánya et al., 2004).

To determine whether emergence of G9 rotaviruses may have been associated with the spread of a single strain or with several distinct strains, studies on the sequence relatedness of G9 VP7 genes of strains from different geographical locations have been conducted. Sequence data to date suggest that the vast majority of currently circulating G9 strains from different locations and different years belong to one closely related phylogenetic lineage of the G9 VP7 gene. This is consistent with the introduction of a single strain, followed by spread and genetic variation in that lineage. These strains are phylogenetically distinct from those reported in the 1980s and also from a few recent isolates, suggesting that a recent common progenitor donated this new G9 VP7 gene. Available sequence and epidemiological data are insufficient to ascertain whether modern serotype G9 human rotavirus strains represent the progeny of a single ancient strain or multiple, independent introductions. With one known exception (strain CIT-254RV; O’Halloran et al., 2002), the serotype G9 VP7 gene is uniformly 1061 bp in length, i.e. 1 nt shorter than most other mammalian rotavirus VP7 genes (Estes & Cohen, 1989). It has been hypothesized that the G9 VP7 gene was generated by a single nucleotide deletion within or at the end of the termination codon of G9 strains (Green et al., 1989; Das et al., 1993). The former hypothesis suggested that the deletion within the termination codon resulted in a new stop codon for G9 strains, UGA, instead of UAG as found in most other human rotavirus G serotypes (Estes & Cohen, 1989; Das et al., 1993). Assuming that this deletion and shift in stop codon usage resulted from a single, ancient molecular event, this finding suggests the early existence of a common ancestor for all or virtually all human rotavirus G9 lineages, including the first isolates from the USA, Japan and India. This observation raises interesting questions about the evolution of the modern lineage from these early lineages and whether these changes occurred in the same host species or accumulated during the course of multiple host switches.

To identify several modern G9 rotavirus lineages that are related to the earliest G9 isolates from the 1980s suggests that most of these strains may represent phylogenetic differences between them.

<table>
<thead>
<tr>
<th>Position</th>
<th>P-like</th>
<th>F45-like</th>
</tr>
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<tbody>
<tr>
<td>87</td>
<td>GGA</td>
<td>G</td>
</tr>
<tr>
<td>105</td>
<td>AAT</td>
<td>N</td>
</tr>
<tr>
<td>126</td>
<td>CCA</td>
<td>P</td>
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<td>141</td>
<td>AGA</td>
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<td>171</td>
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<td>297</td>
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<td>R</td>
</tr>
<tr>
<td>576</td>
<td>AAT</td>
<td>N</td>
</tr>
<tr>
<td>588</td>
<td>TTA</td>
<td>L</td>
</tr>
<tr>
<td>593</td>
<td>GGT</td>
<td>G</td>
</tr>
<tr>
<td>609</td>
<td>ATT</td>
<td>I</td>
</tr>
<tr>
<td>672</td>
<td>AAC</td>
<td>N</td>
</tr>
<tr>
<td>676, 678</td>
<td>CTA/CTG</td>
<td>L</td>
</tr>
</tbody>
</table>

List of exceptions: 1GGG in AI-39; 2GGA in Kagawa/90-554; 3AAT in YO; 4CCG in P and AI-39; 5CCCA in KU and CCT in Kagawa/90-554; 6AGA in AI-39 and KU; 7GAA in RJ28 and IAL28; 8AAG in MW258; 9AAT in BP1744/01; 10CAT in MO; 11TTA in P and AI-39; 12TTG in RJ28 and IAL28; 13AAT in P and AI-39; 14AAT and D in F45; 15ATC in GR1106-86 and GR856-86.
etically distant progeny of those early strains, which may have continued to circulate in humans without detection. In addition to the modern south-east Asian G9 isolates (e.g. 684VN; Nguyen et al., 2001), a number of far-east Asian strains detected in Japan (Zhou et al., 2003) and China (Li et al., 1997) in the late 1990s, or even the predominant, globally distributed modern lineage that is related relatively closely to Mc323-like strains, may support this hypothesis, based on distance and phylogenetic analysis. This hypothesis underscores the importance of continuous and strong genetic drift that has resulted in the segregation of all currently recognized lineages. However, the lack of substantial sequence divergence observed within the predominant modern G9 lineage over a 10 year period contradicts this hypothesis. Thus, the lack of divergence among most modern G9 VP7 genes, together with the observation that substantial differences exist in the genomic constellation of several modern isolates (Laird et al., 2003), suggest that at least some of these lineages may represent independent introductions into the human G9 VP7 gene pool from an unknown source, which could include an animal reservoir.

In fact, early reports demonstrating that some Asian G9 rotavirus isolates were porcine–human or bovine–human reassortants (Urasawa et al., 1992, Das et al., 1993) suggested that genetic interaction between animal and human rotavirus strains may have occurred in the same host. Furthermore, detection of G9 strains in diarrhoeic pigs and the presence of G9-specific antibodies in lambs provide some indirect support for the hypothesis that human G9 rotaviruses could have originated in an animal species (Muñoz et al., 1995; Santos et al., 1999). Additional studies on the genetic relationship between G9 VP7 genes of rotaviruses from animals and humans may shed light on this hypothesis.

Our recent longitudinal, epidemiological survey indicated that serotype G9 rotaviruses were introduced to Hungary more recently, perhaps in the 1997–1998 season (Bányai et al., 2004). In the present study, we have demonstrated the presence of the newly recognized, globally distributed G9 lineage in our country. By using genome-pattern analysis, we have identified four different strains circulating in Hungary over the past few years. Based on previous findings that demonstrated that substantial sequence differences may exist among cognate genome segments of electroherotypically distinct strains (Maunula & von Bonsdorff, 2002), this genomic polymorphism suggests possible changes within the sequences of the corresponding genes. Subsequent sequencing studies have demonstrated that the VP4 gene variable region of E-type B and D strains is genetically distinct from Hungarian P[8],G9 strains of E-types A and C and could be classified into a different lineage of the VP4 gene. This finding suggests that E-type B and D strains acquired a distinct VP4 gene through reassortment, perhaps from one of the epidemiologically common strains (i.e. P[8],G1; P[8],G3; or P[8],G4 rotaviruses), which circulated concurrently with a Hun9-like strain. This mechanism for exchange of VP4 and VP7 genes is well-known amongst epidemiologically common strains (Iturriza-Gómar et al., 2001). It has also been documented through nucleotide sequencing that each of the other nine genes of P[8],G1 strains undergoes frequent reassortment with co-circulating P[8],G4 strains (Maunula & von Bonsdorff, 2002). Hence, determining nucleotide sequences of additional genes, including those that exhibited differences in electrophoretic mobility (e.g. genome segments 2, 3, 4, 5, 6 and 10) in our assay, would probably demonstrate that these strains have undergone frequent reassortment with other co-circulating P[8],G9 strains, as well as with P[8] strains of other G-types. Sequencing of genome segments 6 (encoding VP6) and 10 (encoding NSP4) would have the added advantage that these genes are in genetic linkage and segregate according to the rotavirus host species (Iturriza-Gómar et al., 2003). Thus, analysis of these two genes could allow us to ask whether all Hungarian P[8],G9 strains represent typical human rotaviruses, or whether some may have been derived through reassortment with animal rotaviruses.

Even though we studied a relatively small set of specimens, our findings suggest that the two unique G9 strains with lineage I P[8] specificity (E-types B and D) did not exhibit a high potential for spread during the study period in the areas investigated. In contrast, G9 strains with lineage II P[8] VP4 specificity (E-types A and C) were predominant among P[8],G9 strains. These results are consistent with previous studies that demonstrated that certain genetic lineages within the same VP4 specificity (e.g. within P[8]) are detected infrequently among strains with the same G-types, a finding that may reflect genetic constraints against reassortment for those particular specificities (Iturriza-Gómar et al., 2001).

At present, only a limited number of studies provide supportive data for our hypothesis, particularly in association with P[8],G9 strains. The ‘early’ Japanese P[8],G9 isolate, F45, had VP8 lineage II P[8] specificity (Gouvea et al., 1999), as did a number of P[8],G9 strains that were identified as being epidemiologically important in India (M. Iturriza-Gómar, personal communication), the UK (Iturriza-Gómar et al., 2001), Italy (Martella et al., 2003) and Hungary; however, these latter strains clustered in the same branch within lineage II, distinct from that represented by strain F45. Lineage III P[8] genotype was found in association with serotype G9 only in one study (Iturriza-Gómar et al., 2001). Further studies may elucidate whether the hypothesis that certain lineages are not favoured holds true.

From public health and economic viewpoints, a mass immunization programme against group A rotaviruses is expected to be highly beneficial for both developing and developed countries (Breese et al., 1999). To date, about a dozen different rotavirus vaccine candidates have been or are currently being tested. Many are multivalent vaccines that are composed of animal–human reassortant strains that contain the common human G serotype specificities G1–G4 and, in one case, the most common P serotype specificity, P[1]A[8] (Midhun et al., 1985; Clements-Mann et al., 2001). All of these vaccines were developed based in part on the percep-
tions that animal strains are in general naturally attenuated for humans and that serotype-specific immunity is important to prevent severe (re-)infections. RRV-TV (Rotashield) met these criteria but, due to an association with intussusception after its US introduction, it was withdrawn, leaving several questions unanswered. One important issue that remained to be studied was the extent of cross-protection raised by multivalent vaccines against serotypes that were not present in the vaccine. Thus, the emergence of strains previously considered uncommon (e.g. serotype G9) may have serious implications for future vaccination programmes.

In some early reports, infections with the novel G9 strains were associated with unusual epidemiological and clinical features. British investigators (Iturriza-Gómar et al., 2000) found that recently emerged G9 strains caused symptomatic infections in children over 5 years of age, where pre-existing immunity was expected to provide sufficient heterotypic protection against common and, perhaps, rare serotypes. Another study reported that G9 strains caused disease in newborns, suggesting that acquired maternal antibodies did not neutralize these strains efficiently (Widdowson et al., 2000). Both studies suggested that G9 rotaviruses escaped the pre-existing immunity evoked by other strains, possibly because these populations were immunologically naive to G9 specificity.

These findings emphasize the importance of serotype-specific immunity and support the hypothesis that an effective vaccine should represent all specificities that occur locally. Thus, if serotype G9 rotaviruses continue to be a common cause of diarrhoea in children, despite the rise of population immunity to these strains, it might be necessary to add G9 VP7 specificity to future multivalent vaccine candidates. Therefore, it will be important to monitor the ability of vaccines that are currently undergoing trials to provide heterotypic protection against G9 strains.

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