Molecular Epidemiology of Rotavirus in Central and Southeastern Europe

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A surveillance network was implemented by the Istituto Superiore di Sanità of Rome in collaboration with laboratories of virology in Czech Republic, Slovenia, Croatia, Albania, and Bulgaria. About 1,500 rotavirus-positive stool samples were collected from children with severe gastroenteritis admitted to hospitals or outpatient wards between 2004 and 2006. The G and P genotypes were determined by reverse transcription–nested PCR. Significant differences were found in the geographical distributions of rotavirus genotypes between countries participating in the study. The prevalence of “common” G/P combinations, G1P[8], G3P[8], G4P[8], and G2P[4], ranged between 50 and 85%. The G9 genotype, which is emerging worldwide, was identified in 2 to 35% of all samples depending on the country. Unusual combinations, such as G1 or G4 associated with P[4] or G2 with P[8], which may have arisen by reassortment between human strains, were found in samples from 3 to 20% of patients. The uncommon genotypes G8P[8] and G10P[6], which may have an animal origin, were also identified. Double infections with two rotavirus strains were observed in between 1.7 and 14% of cases studied. Our findings might implicate challenges for rotavirus vaccine implementation in a wide geographic area of the Balkans and Central-Eastern Europe and underscore the importance of extensive strain surveillance for success in vaccine development.

Human group A rotaviruses are the most frequently identified etiologic agents of acute gastroenteritis in infants and children worldwide. Each year, rotavirus causes approximately 111 million episodes of gastroenteritis requiring only home care, 25 million clinic visits, and 2 million hospitalizations (37). About 600,000 children die following rotavirus infection every year, mainly in developing countries (19).

Rotavirus is characterized by three protein shells (identifying an outer capsid, an inner capsid, and the core) that surround 11 segments of double-stranded RNA (3). The outer layer of rotaviruses is composed of two proteins, the glycoprotein VP7 (G protein) and VP4, a protease-cleaved protein (P protein). These proteins elicit neutralizing antibody responses and are implicated in the current dual classification of group A rotaviruses into G and P types. Genes encoding the G and P proteins can be characterized by reverse transcription-PCR (RT-PCR), and 10 different G genotypes and 11 P genotypes have been identified among strains found in human infections (29). Additional G and P types have been found in animal species only (18).

The introduction of molecular typing methods has enhanced our understanding of the diversity of rotavirus strains, which affects the development of rotavirus vaccines and involves viral evolution. Four common G types (G1P[8], G2P[4], G3P[8], and G4P[8]) represent more than 88% of the strains analyzed worldwide (43). These strains have become the most important targets for vaccine development. When the global G- and/or P-type distributions were divided into five continents/subcontinents, several characteristic features emerged (43). Compared to the developed world, the diversity of unusual strains in developing countries is great and more strains circulate at any time, and as many as 30% of children can be infected with more than one strain during a single episode of infection. In addition, prevalent rotavirus genotypes detected in different areas of the world may be completely different.

A number of studies demonstrated the importance of serotypes other than G1 to G4 as a cause of gastroenteritis in children. These studies included the detection of G5P[8] in Brazil, G8P[6] and G8P[4] in Malawi, and G9P[6] in India (14, 21, 32, 39). In addition, G9 strains have recently entered the human population, not only in the tropics but also in countries with temperate climates, such as the United States, Australia, Ireland, Japan, Italy, the United Kingdom, and France (40).

The finding of this enormous diversity among strains provides insights into rotavirus evolution. For human rotaviruses, three major evolutionary mechanisms have been identified:
point mutations, occurring as singular events or accumulating sequentially over time; genomic reassortment in the progeny of two viruses after coinfection of a single cell; and the introduction of animal rotaviruses into the human population. These mechanisms contribute to the diversity of rotavirus strains individually and in combination (18, 26, 36).

The enormous diversity and capacity for change of human rotaviruses suggest that rotavirus vaccines must provide good heterotypic protection to be optimally effective (18). Two vaccines from GlaxoSmithKline (Rotarix) and Merck (RotaTeq) have been licensed in Europe and the United States, respectively, and in several other countries (19). Rotarix was developed from the naturally attenuated rotavirus strain 89-12 and represents the most common serotype in humans (P[8], G1) (8). RotaTeq contains five human-bovine reassortant rotaviruses (G1, G2, G3, G4, and P[8]) (23). The extent to which these rotavirus vaccines will induce heterotypic immunity is not yet established. Therefore, rotavirus strain surveillance appears to be needed before and after the introduction of new vaccines in order to monitor the actual prevalence and possible changes of the different G and P types circulating in different areas. Studies should also include detection of uncommon and novel types of virus which might cause vaccine failure. The emergence of new and immunologically distinct rotaviruses, possibly through the transmission of viruses across species barriers or reassortment between animal and human rotaviruses, may make it necessary to modify vaccines from time to time (15, 25).

We report here the results of 2 years of rotavirus strain surveillance with children with severe gastroenteritis admitted to hospitals or outpatient wards in five countries of Central and Southeastern Europe. Our goals were to describe in detail the molecular epidemiology of rotavirus infections and to monitor the emergence and spread of novel rotavirus strains in selected regions. This was the first time, the surveillance network was established in such a wide geographic area of the Balkans and Central-Eastern Europe. The same system could be used in the future to monitor the impact of a vaccination effort.

**MATERIALS AND METHODS**

**Rotavirus study group.** A surveillance network was organized by the Istituto Superiore di Sanità of Rome in collaboration with laboratories of virology in the Czech Republic, Slovenia, Croatia, Albania, and Bulgaria. The whole list of collaborating laboratories and hospitals is given in Table 1. The hospitals were mostly located in big cities or capitals.

**Patients and samples.** Stool samples were collected from children with severe gastroenteritis, mostly of ages less than 5 years, admitted to hospitals or outpatient wards in five countries of Central and Southeastern Europe. Our goals were to describe in detail the molecular epidemiology of rotavirus infections and to monitor the emergence and spread of novel rotavirus strains in selected regions. This was the first time, the surveillance network was established in such a wide geographic area of the Balkans and Central-Eastern Europe. The same system could be used in the future to monitor the impact of a vaccination effort.

**Rotavirus testing.** Stool specimens were collected on admission and either analyzed immediately after collection or stored at −20°C until processing.

Screening of stools was performed with the commercially available latex agglutination test Rotalex or Diarlex Rota-Adeno (Orion Diagnostica) or an enzyme-linked immunosorbent assay (ELISA) test for rotavirus antigen detection. Overall, approximately 1,500 rotavirus-positive stools were collected, ranging from the 657 samples of Slovenia to the 71 of Bulgaria (Table 1). After identification of group A rotaviruses by the local laboratory, positive stools were shipped in dry ice to the Istituto Superiore di Sanità of Rome for strain characterization. All samples arrived in good condition, and the cold chain was maintained. A 10% suspension in water was prepared for further tests by rotavirus-specific RT-PCR and nested PCR.

**Nucleic acid extraction and G/P rotavirus typing.** Double-stranded viral RNA was extracted from 140 μl of the 10% fecal suspension by using a commercial kit (QIAamp viral RNA minikit; QIAGEN) according to the manufacturer’s instructions. RNA was eluted in 50 μl of RNase-free water and stored at −80°C. Next, G and P rotavirus genotyping was performed by using RT-PCR methods as previously reported (22).

For the identification of the G type, the VP7-F and VP7-R consensus primers (20) were used in RT-PCR. Subsequently the VP7-R primer was used in a nested multiplex PCR together with G1, G2, G3, G4, G8, G9, and G10 typing (20, 22, 27). Samples testing negative by G typing with the primers described above were also tested by using a PCR with G12-specific primers (9, 41). Con2-Con3 consensus primers (17) were used in RT-PCR for P types, followed by the standard multiplex PCR including the Con3, in combination with typing primers P4, P6, P8, P9, P10, and P11 (17, 24, 27). VP6 RT-PCR was performed as previously reported (28).

**DNA sequencing and analysis.** Some of the rotavirus strains were characterized by nucleotide sequencing of the amplified PCR products. These products were purified by using the Wizard SV Gel and PCR Clean-Up System (Promega) and then sequenced using an ABI sequencer apparatus (model ABI3730XL; Applied Biosystems). Data analysis and multiple alignments were performed by using DNASIS MAX v.2. The sequences were compared against the GenBank database using the BLAST program.

**RESULTS**

**Prevalence of rotavirus G and P types.** A total of 1,528 rotavirus strains from Croatia, Slovenia, the Czech Republic, Albania, and Bulgaria were G and P genotyped. The overall results, including mixed infections with multiple rotavirus strains, are shown in Table 2, where data are presented by country of origin. Among the VP7 genotypes identified, genotype G1 was predominant in Slovenia (54.8% of all cases), the Czech Republic (40.4%), and to a lesser extent in Croatia (24.2%). Conversely, G4 was the predominant rotavirus genotype in Albania (46.2%) and Bulgaria (38.0%). Viruses belonging to the G2 type were found in a lower percentage of cases (range, 3.0 to 11.5%) in all countries with the exception of Croatia, where the prevalence reached 23.1% of the total. The G3 type was detected only in Croatia and the Czech Republic, with rates close to 5%. The G8 genotype was observed in 8.5% of cases in Croatia, and it was not detected in any other areas investigated with the exception of one sample from Slovenia. The emerging G9 genotype occurred in several countries with

<table>
<thead>
<tr>
<th>Table 1. Rotavirus surveillance network</th>
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<tbody>
<tr>
<td>Country</td>
</tr>
<tr>
<td>Slovenia</td>
</tr>
<tr>
<td>Croatia</td>
</tr>
<tr>
<td>Czech Republic</td>
</tr>
<tr>
<td>Albania</td>
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<tr>
<td>Bulgaria</td>
</tr>
</tbody>
</table>

* n, no. of samples provided.
a range between 2.2 and 11.1% of cases, whereas it showed a particularly high frequency in Bulgaria (36.6%), where it was virtually as common as the predominant G4 type found in that country. G10 strains were rarely identified in all countries studied.

Among the VP4 genotypes, P[8] occurred most frequently (up to 80%) in all countries, followed by P[4] (up to 35%), P[1], P[6], and P[11] were rarely identified in Croatia and Slovenia.

Strains that could not be genotyped by G- or P-typing PCR or both are also shown in Table 2. The G or P type could not be assigned in 2.8 to 13.1% or 11.3 to 18.7% of cases, respectively.

Combinations of G and P types of the rotavirus strains. Among the rotavirus strains identified in the present investigation, a number of different G and P combinations were detected, some of which were very frequent while others occurred only rarely. These data are shown in Table 3, where P/G combinations are divided into “common” or “uncommon” based on established knowledge and previous reports. The former group, which included G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8], altogether accounted for a percentage of 82.6%, 77.7%, and 71.2% in Bulgaria, Czech Republic and Slovenia, respectively. In Croatia and Albania, the prevalence of “common” P/G combinations was lower, i.e., 59.9 and 50.0%, respectively. Within the latter group, each single G/P combination occurred to a different extent among countries, in agreement with the different distribution of single G and P types reported before (see Table 2). Overall, G1P[8] was the virus most frequently observed (32.1%), followed by G4P[8] and G2P[4] (18.5 and 8.1%, respectively), whereas G9P[8] and G3P[8] were detected more rarely (5.2 and 2.2%, respectively). G1P[8] rotaviruses were found to represent only 8.9 and 7.1% of strains typed in Albania and Bulgaria, where this otherwise frequent genotype was replaced as the predominant circulating rotavirus strain by G4P[8] or both G4P[8] and G9P[8] viruses, respectively. In Albania, the decline of G1P[8] was balanced by the spread of viruses with “uncommon combinations”, which overall were present in as high as 23.4% of cases. Among these, G4P[4] was detected in 13.3% of all cases, but G1P[4] and G2P[8] were also relatively frequent (4.4 and 5.7%). “Uncommon” G/P combinations were also found in the other four countries, ranging from 2.8% in Slovenia to 8.0% in Croatia, where the unusual G8P[8] type was identified in 1 and 31 cases, respectively. The involvement of G8 rotavirus in the infections was particular high in the second epidemiological season in Croatia (6.8% of all strains).

Double infections with different strains of rotavirus were also detected in a range between 1.6 and 12.7% of patients from the different countries, with a particularly high frequency in Albania. The occurrence of uncommon types of rotavirus appeared to be correlated with the prevalence of mixed infections in the different countries (Fig. 1).

The untypeable strains occurred at significantly different frequencies between countries (0 to 3.5% for the samples with untypeable G and 3.9 to 11.4% for the samples with untypeable P). The frequency of untypeable G strains was higher in Croatia, whereas untypeable P strains were found mostly in Slovenia. For 2.9 to 9.6% of the cases, neither G nor P typing could be obtained with the methods described above. How-
ever, in about 30% of these last cases, the presence of rotavirus in the samples was confirmed by the observation of a DNA band of the expected size after G and/or P first-round RT-PCR or by a positive VP6 RT-PCR.

Sequence analysis. Selected of particular interest samples (total number /H1100530/) were further analyzed by nucleotide sequencing of the amplified band after G- and/or P-typing PCR. Comparison of sequences revealed a close relationship between the G9 VP7 genes of strains found in our study and most of the G9 strains recently identified in European, American, African, and Asian countries (4, 6, 33, 44, 49) (96 to 99% sequence similarity; data not shown).

Sequence analysis of the G8 strains from Croatia showed a very close homology (more than 98%; data not shown) to other human G8 strains recently isolated in Slovenia (GenBank accession number DQ995179) and previously in Malawi (13). A high sequence homology of these strains with bovine G8 rotaviruses, such as the G8 strain detected in Nigeria (2) (homology, 98%; data not shown) was also detected, which suggests a possible human/animal viral reassortant.

Temporal distribution of G rotavirus genotypes. In all countries except Albania, samples were collected from ill children during the two consecutive rotavirus epidemic peaks in winter-spring 2005 and 2006, which made it possible to analyze changes with time for rotaviruses in these countries. In Albania, stools were collected in 2004 and 2005, and it was not possible to obtain samples in 2006. For this country, only one overlapping year was used for comparison. The analysis of genotypes circulating with time did not show remarkable differences in the predominant G types in the Czech Republic and Slovenia, while a marked shift was observed in other countries. In particular, G9 and G8 types spread widely during the 2006 rotavirus epidemics in Bulgaria and Croatia, respectively, whereas they were present to a much lesser extent or even absent during 2005, when the common strains G1, G2, and G4 were predominant in these two countries (Table 2). Besides the emergence of G9 and G8 genotypes during the second rotavirus season in Bulgaria and Croatia, we also detected a higher percentage of mixed infections and nontypeable strains in these countries.

Table 3. G- and P-type combinations detected in 1,528 rotavirus strains from Croatia, Slovenia, the Czech Republic, Albania, and Bulgaria during a 2-year perioda

<table>
<thead>
<tr>
<th>G/P combination type</th>
<th>Slovenia</th>
<th>Croatia</th>
<th>Czech Rep.</th>
<th>Albania</th>
<th>Bulgaria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common types</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1P[8]</td>
<td>304 (46.3)</td>
<td>100 (21.8)</td>
<td>66 (35.1)</td>
<td>14 (8.9)</td>
<td>5 (7.1)</td>
</tr>
<tr>
<td>G2P[4]</td>
<td>7 (1.1)</td>
<td>88 (19.2)</td>
<td>11 (5.9)</td>
<td>9 (5.7)</td>
<td>3 (4.3)</td>
</tr>
<tr>
<td>G3P[8]</td>
<td>23 (5.0)</td>
<td>9 (4.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G4P[8]</td>
<td>93 (14.2)</td>
<td>58 (12.6)</td>
<td>47 (25.0)</td>
<td>51 (32.3)</td>
<td>26 (37.1)</td>
</tr>
<tr>
<td>G9P[8]</td>
<td>64 (9.7)</td>
<td>6 (1.3)</td>
<td>13 (6.9)</td>
<td>5 (3.2)</td>
<td>24 (34.3)</td>
</tr>
<tr>
<td>Uncommon types</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1P[4]</td>
<td>1 (0.2)</td>
<td>11 (2.4)</td>
<td>3 (1.6)</td>
<td>7 (4.4)</td>
<td></td>
</tr>
<tr>
<td>G2P[8]</td>
<td>11 (1.7)</td>
<td>17 (3.7)</td>
<td>6 (3.2)</td>
<td>9 (5.7)</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td>G4P[4]</td>
<td>1 (0.2)</td>
<td>8 (1.7)</td>
<td>1 (0.5)</td>
<td>21 (13.3)</td>
<td></td>
</tr>
<tr>
<td>G9P[4]</td>
<td>3 (0.5)</td>
<td>1 (0.2)</td>
<td>1 (0.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G8P[8]</td>
<td>1 (0.2)</td>
<td>31 (6.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G10P[6]</td>
<td>(0.0)</td>
<td>1 (0.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G10P[8]</td>
<td>1 (0.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G10P[9]</td>
<td>1 (0.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mixed infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dual G type</td>
<td>25 (3.8)</td>
<td>32 (7.0)</td>
<td>5 (2.7)</td>
<td>20.0 (12.7)</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td>Dual P type</td>
<td>6 (0.9)</td>
<td>2 (0.4)</td>
<td>1 (0.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dual G and P</td>
<td>1 (0.2)</td>
<td>3 (0.7)</td>
<td>1 (1.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontypeable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GntPpos b</td>
<td>15 (2.3)</td>
<td>16 (3.5)</td>
<td>2 (1.1)</td>
<td>1 (0.6)</td>
<td></td>
</tr>
<tr>
<td>GposPnt c</td>
<td>75 (11.4)</td>
<td>18 (3.9)</td>
<td>15 (8.0)</td>
<td>6 (3.8)</td>
<td>6 (8.6)</td>
</tr>
<tr>
<td>GntPnt d</td>
<td>48 (7.3)</td>
<td>44 (9.6)</td>
<td>8 (4.3)</td>
<td>15 (9.5)</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td>Total</td>
<td>657</td>
<td>459</td>
<td>188</td>
<td>158</td>
<td>71</td>
</tr>
</tbody>
</table>


b GntPpos, G nontypeable but with identified P type.
c GposPnt, P nontypeable but with identified G type.
d GntPnt, both G and P nontypeable.

e Czech Republic.
more frequent (13.9% and 11.7%, respectively) during the first season (Table 2).

**Distribution of rotavirus genotypes by age.** The age distribution analysis of the patients confirmed to be rotavirus positive showed that most of the rotavirus infections occurred in children under 3 years of age, with a peak incidence between the ages of 1 and 2 years (34.1% of all cases). Overall, 10.6% of patients were younger than 1 year, 24.7% of patients were between 2 and 3 year old, 20.7% of all cases were in the age group of 3 to 5 years, and 9.9% were found in children of ages greater than 5 years or in adults.

The G9 and G8 strains which emerged in Bulgaria and Croatia, respectively, during the 2006 epidemics were more common among younger children than other G types. In fact, 59% of G9 rotavirus infections in Bulgaria and 38% of G8 rotavirus infections in Croatia were found in infants less than 1 year old, who represented only 25 and 17% of the corresponding populations studied.

**DISCUSSION**

To our knowledge, this molecular epidemiological study of rotavirus strains circulating in the Czech Republic, Slovenia, Croatia, Albania, and Bulgaria is the most systematic study of rotavirus diversity in such a wide geographic area of the Balkans and Central-Eastern Europe.

Previous local studies in this region were in fact conducted only in single countries and identified G9 rotavirus as the predominant type in Slovenia during the 2001-2002 winter season (45) and G1 in the Czech Republic during 1999 to 2002 (38) and identified both G9P[8] and G3P[8] rotaviruses as being responsible for the outbreak at the end of December 2000 in Albania (48).

The simultaneous investigation of five countries during two full years may be very useful for evaluating the impact of the approaching mass vaccination of children in Europe and other countries. In fact, a monovalent live attenuated vaccine with a G1P[8] genotype was recently approved by the European Medicines Agency, and a pentavalent G1-4P[8] vaccine has been approved by the Food and Drug Administration in the United States and is approaching registration in the European Union as well. Although both vaccines have been claimed to induce a broad-spectrum immunity towards heterologous rotavirus serotypes (7, 47), knowledge of the shift of predominant rotavirus strains and emergence of novel genotype combinations may address the present congruity of the vaccine formula and future needs for its updating.

Altogether our surveillance data demonstrate the great diversity of G and P genotypes of rotavirus circulating in the same period in a given population. The prevalence of a “common” G/P combination, G1P[8], G4P[8], G2P[4], G3P[4], and G9P[8], varied in our study from 50 to 83%. Normally, these combinations represent >90% of the rotavirus infections in Europe (43). The low prevalence of “common” P/G combinations in Croatia and Albania (59.9 and 50%, respectively) is a situation typical for countries of the tropics and subtropics and is quite unusual for temperate climates (15, 43). Possibly the circulation of unusual rotavirus types in European countries could partly be explained by the immigration flow, which uses part of the Balkan border as a port of entry to the European Community. For instance, G8 genotypes have been previously reported in Africa (2, 14).

In the past several years, the G9 serotype has emerged as the fifth common type worldwide, including in Europe (4, 6, 31, 33, 35, 42–44, 48). In our study, G9 rotavirus strains were generally identified at relatively low rates (Table 2), ranging from the 1.5% to 10.2%. The only exception was Bulgaria, where the prevalence of G9 strains was overall 34.3% and was as high as almost 60% of all cases detected during the epidemic peak of 2006. Analysis of nucleotide sequences of representative G9 strains found in this study revealed in all cases a close relationship to most G9 strains recently identified in European, American, African, and Asian countries (4, 6, 33, 44, 49), which are more closely related to each other than to earlier G9 strains isolated in the 1980s (31). The surveillance of G9 strains is particularly important, because the degree of protection of current vaccines against this serotype is yet to be confirmed. In the future, the G9 component may need to be included in the formulation of an effective rotavirus vaccine, especially considering the great ability of these strains to spread widely, as was the case for Bulgaria in this study.

Another interesting finding is the appearance of the G8 strains observed in Croatia in 2006, which were not present among Croatian strains investigated during the 2005 peak. Besides the serotype being a novel one for the area, the emergence of G8 strains is also worth mentioning because G8 rotaviruses are likely to be of animal origin. In fact, the high sequence homology of the presently described G8 strains with other human and bovine G8 rotaviruses (2, 13) confirms this possibility and reinforces the hypothesis of domestic animals as a possibly relevant reservoir of rotavirus for humans (11, 34). It is not known whether infection with G8 rotaviruses could be efficiently prevented by vaccination with G1-to-G4 serotype-based vaccines, but the large diffusion of G8 and other atypical serotypes in developing countries might indeed be involved in the lower efficiency of live rotavirus vaccination trials experienced in those areas. Previous studies on rotavirus G and P
typing show that in the great majority of cases reported for countries of temperate climates (43), G1, G3, and G4 are commonly associated with P[8] and G2 is associated with the P[4] type. In our study, we detected many strains showing the “uncommon” G/P combination G1P[4], G2P[4], or G4P[4], the latter with a particularly high rate in Albania (13.3% of all cases [Table 3]). These data further suggest that reasortment between common strains of rotavirus may occur easily in nature, as also has been documented in previous surveillance studies (1, 18, 26, 36).

The peak incidence for G9 and G8 strains was observed in infants less than 1 year old, whereas the peak incidence for all rotavirus infection, including common G1 to G4 strains, was observed for the age group 2 to 3 years. These results might suggest the absence of maternal neutralizing antibodies specific for the G9 and G8 strains, recently introduced in Europe. Nonetheless, it is not obvious why older subjects would prove relatively more immune to these emerging serotypes than they actually were against common rotavirus serotypes. An increased risk of G9 infection in younger infants was also found in other studies (3, 10), although Cubitt and colleagues (12) reported a higher incidence of G9 strains in older children. This apparent inconsistency could be explained by the existence of some heterotypic protection versus G9P[8] and G8P[8] strains in older children, already immune to the other more common serotypes. In fact, the role of the widely spread P[8] genotype among human rotavirus strains might be much more important than is usually considered and might be a key factor in the immunity conferred by the monovalent glycogen synthase kinase G1P[8] vaccine towards G1 to G4 and G9 strains. As a matter of fact, this vaccine appears to show a relatively lower efficacy against G2P[4] infection (30). On the other hand, it cannot be ruled out that some older children could have already been infected with G9 rotaviruses at younger ages. In fact, we have epidemiological data only about the circulation of G9 strains in Albania in 2000 and in Slovenia in 2001 and 2002 (45, 48), but we do not know whether the G9 serotype had emerged previously in these and other countries included in this study.

Desselberger and colleagues (15) recently reviewed data from different earlier studies, showing that although G1 to G4 rotavirus types as a whole have constantly predominated in Europe, the specific type distribution can vary over time (5, 15). Similar changes in the relative frequencies of “common” genotypes with time were also seen in all countries investigated by us, which culminated in a shift of the predominant serotype from G1 to G2, from G4 to G1, and from G4 to G9 in the cases of Croatia, Albania, and Bulgaria, respectively, between the two years studied. The emergence of G9 and G8 genotypes during the second rotavirus season, respectively, in Bulgaria and Croatia was accompanied by a remarkably high occurrence of either mixed infections or nonsubtypeable strains compared to the previous season. A similar phenomenon has been reported to have occurred in Spain during 2005 (42), when the G9 genotype became predominant (50.6%) and relatively high rates of multiple infections (11.4%) were simultaneously detected.

Depending on the country, we have detected mixed infections with different strains of rotavirus in 1.6 to 12.7% of all patients, with the latter figure representing quite a high frequency for Europe. In fact, a study conducted in United Kingdom (25) found that only 2.2% of all samples contained mixed genotypes, and 6% of mixed infections were shown in Spain (42). Only two studies, in Denmark (16) and Albania (48), reported rates of mixed rotavirus infections even higher than the present case (21% and 42.8%, respectively). In contrast to the case in industrialized countries, high levels of mixed infections are often present in developing countries, where greater variation of different genotype combinations is also usually detected (18).

In our study, the highest percentages of mixed infections were observed in Albania (12.7%) and Croatia (8.1%), which also showed the highest percentages (23.4% and 8.1%, respectively) of rotavirus strains with uncommon genotype combinations (Table 3). Overall, the percent values of all uncommon rotavirus strains (Fig. 1), which are likely to be natural reassortants, and the percentages of all mixed infections for each single country correlated well ($r = 0.92$). This finding suggests that the level of mixed infections may determine the level of natural reassortment events, playing an important role in generating rotavirus strain diversity.

The number of untypeable samples was unusually high in our study, especially in the cases of Croatia and Slovenia (Table 3), where we were unable to determine one or both G/P types in up to 18.7% of samples. Having used a latex agglutination test and ELISA for screening of positive rotavirus samples, it is reasonable to assume that 3 to 5% (25) of the G/P double-negative samples could be false positive. However, the number of double untypeable strains in our case (2.9 to 9.6%, depending on the country) was too high to assume this as the only explanation. Seventy percent of samples with nested PCRs negative for both G and P types were also negative by VP6 RT-PCR. In approximately 30% of these, we found evidence of a possible presence of rotavirus antigen, using a sensitive in-house ELISA (not shown), but in the absence of amplified DNA we cannot confirm detection. Overall, these results suggest the circulation of viruses with unusual G and/or P genotypes which are not detected by the sets of primers normally adopted in genotyping protocols designed for human strains. Some of the untyped strains may indeed be strains of animal origin, such as G5, G6, P[1], P[5], and P[7], which we have not specifically searched for, or human strains mutated at the genotype-specific primer binding site through genetic drift.

The introduction of new viruses or genes into the pool of rotavirus affecting humans must be taken into account for continuing implementation of rotavirus vaccines. Reduction of the intolerable mortality in developing countries is believed to represent an urgent need, but even if mortality caused by rotavirus in Europe and other developed areas is low, deaths of otherwise healthy infants are not acceptable either, especially when they are preventable by vaccination (46). Rotavirus strain surveillance conducted before and after the introduction of vaccination appears thus to be needed in order to understand if vaccines can provide adequate protection against heterologous strains not included in vaccines and to monitor the potential effects of vaccination on serotype prevalence and circulation. Detection of uncommon and novel types of rotavirus, particularly strains emerging by reassortment between animal...
and human rotaviruses, is critical to ensure suitability of human rotavirus vaccines over time (15).

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