Molecular and Clinical Characterization of Rotavirus From Diarrheal Infants Admitted to Pediatric Emergency Units in France

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Background: Rotaviruses are the major cause of acute gastroenteritis in young children worldwide, and require careful surveillance, especially in the context of vaccination programs. Prospective surveillance is required to monitor and characterize rotavirus infections, including viral and clinical data, and to detect the emergence of potentially epidemic strains.

Methods: Between 2006 and 2009, stool samples and clinical records were collected from 2044 children with acute diarrhea admitted to the pediatric emergency units of 13 French university hospitals. Rotaviruses were detected in stools, then genotyped by reverse transcription-polymerase chain reaction with regard to their outer capsid proteins VP4 and VP7.

Results: The genotyping of 1947 rotaviruses showed that G1 (61.7%) and G9 (27.4%) strains were predominant and stable, followed by G2 (6.5%), G3 (4.0%), and G4 (2.5%) strains. Most strains were associated with P[8] (92.9%). Overall, 31 uncommon strains and possible zoonotic reassortants were detected including G12 and G8 strains, some being closely related to bovine strains. No difference in clinical presentation and severity was found among genotypes.

Conclusions: The relative stability of rotavirus genotypes currently cocirculating in France may ensure vaccine effectiveness in the short and medium term. However, the likely emergence of G12 and G8 strains should be monitored during ongoing and future vaccination programs, especially as all genotypes can cause severe infections. Special attention should be paid to the emergence of new rotavirus reassortants not included in current rotavirus vaccines.

Key Words: rotavirus, diarrhea, acute gastroenteritis, children, genotype, severity

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Today rotavirus diarrhea is still one of the main causes of morbidity and mortality in infants throughout the world, and combating this disease burden has become a global public health priority that hinges on programs to develop vaccines. Rotavirus infections have a wide spectrum of clinical manifestations ranging from asymptomatic infections to severe and lethal cases of diarrheal disease. Although fast and appropriate care has considerably reduced mortality in the western countries, rotaviruses are still responsible for considerable morbidity in infants[1,2] and generate significant health costs.[3,4]

On the basis of the outer capsid proteins VP4 (P-types) and VP7 (G-types), group A rotaviruses can be classified into 31 P and 19 G genotypes,[5,6] thus showing a considerable diversity of strains. Both viral outer layer proteins elicit the production of neutralizing antibodies in the host,[7] but no precise G or P type clearly correlates with the severity of the disease. Five rotavirus genotypes G1 to G4 and G9 are responsible for approximately 90% of the diarrhea episodes in children.[8,9] Of note, uncommon rotavirus G genotypes such as G5, G8, G10, and G12 are emerging in some areas of the world, notably in tropical regions.[9] Taking into account the antigenic diversity of rotaviruses, a variety of approaches toward the development of effective rotavirus vaccines have been undertaken. Currently, 2 live-attenuated oral vaccines have been licensed in many countries of which France and were found to be efficacious against most circulating rotaviruses[10–12]: a pentavalent bovine-derived rotavirus vaccine (RotaTeq, Sanofi-Pasteur-MSD) and a monovalent human-derived rotavirus vaccine (Rotarix, GSK). Of note, vaccine coverage is currently less than 10% in infants in France.

Local data on the current burden of rotavirus disease, investigating both virologic and clinical aspects, are important for decisions and optimizations regarding immunization strategies.[13,14] Hence, the search for the determinants of the clinical outcomes, including the possible role of the different rotavirus genotypes, and the knowledge of molecular epidemiology and

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antigenic diversity of cocirculating rotaviruses are necessary to ensure the suitability and the efficacy of the both vaccines. Indeed, rotavirus diversity is constantly generated by positive selection of single amino acid mutations in defined epitopes, and particularly in highly divergent regions of the outer capsid protein VP7.[15,16] Programs for monitoring of rotavirus antigenic drifts that may be caused by specific immunologic pressures and the monitoring of potential reassortments between human or human and animal rotavirus strains have to be carefully developed.

Since the establishment of a national rotavirus surveillance in France in the mid-2000s, it has become possible to assess the nationwide circulation of rotavirus strains, especially in infants. This prospective study was designed to monitor and characterize rotavirus infections, including both viral and clinical data, in children under 5 years old suffering from community-acquired acute gastroenteritis and admitted to pediatric emergency units during rotavirus vaccine introduction in France. Special attention was paid to the detection of uncommon strains and emerging reassortants that may not respond to current rotavirus vaccines.

MATERIALS AND METHODS

Study Design

After its approval by the local ethical committee, the surveillance study was conducted during 3 consecutive seasons from July 2006 to June 2009, and involved 2044 children under 5 years suffering from rotavirus-induced acute gastroenteritis and admitted to the pediatric emergency units of 13 French University Hospitals: 3 in Paris and 10 in various provincial regions (Fig. 1).

Acute gastroenteritis was defined by at least 3 soft or liquid stools or 3 bouts of vomiting in 24 hours or one of the following signs: diarrhea and/or vomiting accompanied by at least 2 additional symptoms: abdominal pain or fever. Children presenting with chronic diarrhea, immune deficiency, inflammatory disease of the digestive tract, or nosocomial infections were excluded.

Clinical data, including personal identification, rotavirus vaccination status, clinical symptoms such as daily numbers of stools and bouts of vomiting, fever and blood in stool, and severity criteria such as the degree of dehydration, oral or intravenous rehydration, duration of the hospitalization including journey in intensive care unit, and death were collected from children admitted to pediatric departments.

Sample Analysis Methods

The stool samples were routinely screened for group A rotavirus using enzyme immunoassays (EIA) tests as previously described.[17] All rotavirus-positive samples were confirmed by reverse transcription polymerase chain reaction (RT-PCR) and genotyped using hemi-nested multiplex RT-PCR as previously described.[18–23] The PCR products of representative strains and uncommon strains were purified and sequenced as previously described.[17] The nucleotide sequences were edited and typed as described previously using BioNumerics software (Applied Maths NV, Sint-Martens-Latem, Belgium). For uncommon strains, addi-

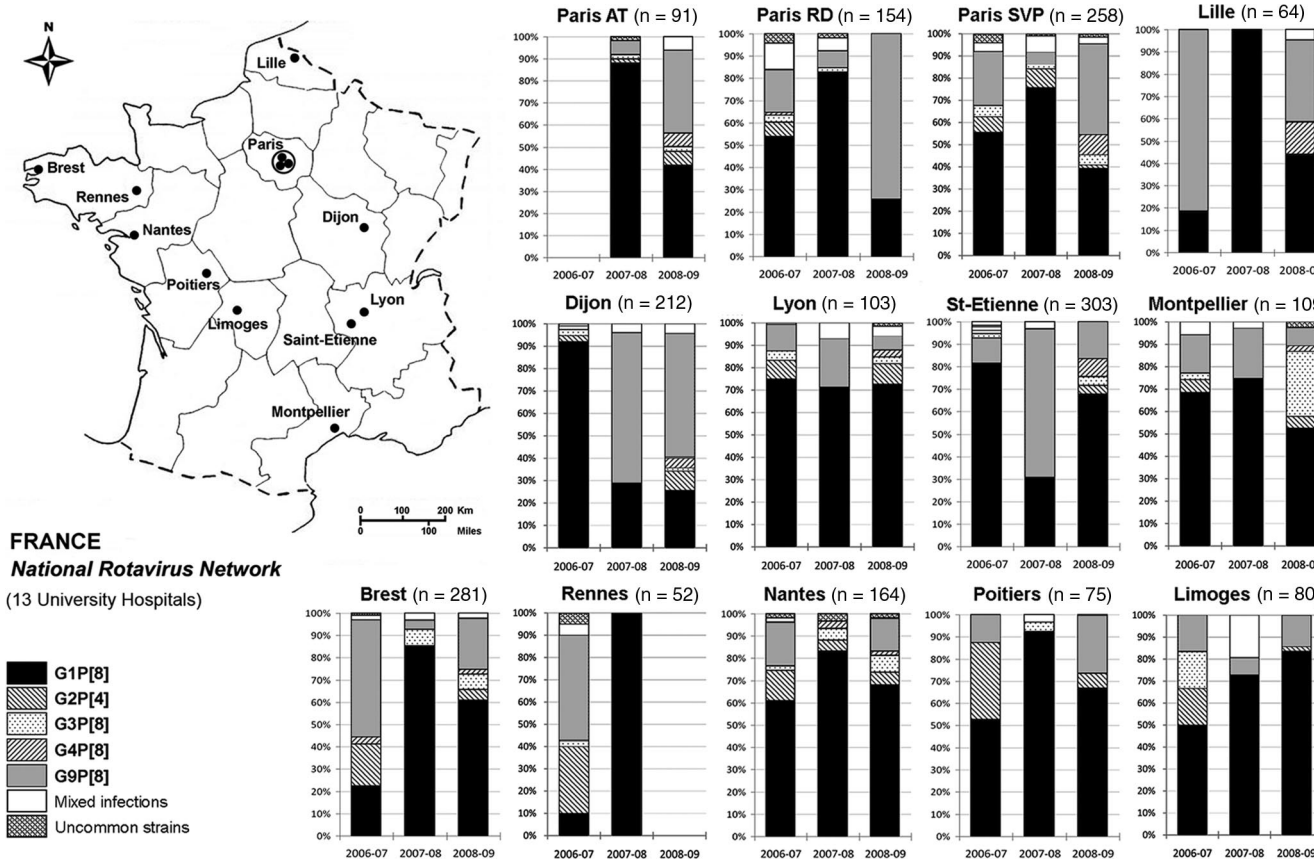

FIGURE 1. Map of the French national rotavirus network showing the seasonal distribution of rotavirus genotypes in each center between 2006 and 2009. AT indicates Armand-Trousseau Hospital, Paris; RD, Robert-Debré Hospital, Paris; SVP, Saint-Vincent-de-Paul Hospital, Paris.

tional analyses were performed for the VP6 and NSP4 genes following the same procedures and using methods previously described.[24,25] The nucleotide sequences of representative strains have been deposited in GenBank.

Statistical Analysis Methods

Statistical analyses were performed using STATA v11.0 software from StataCorp (College Station, TX). Comparisons were performed using the Pearson χ^2 (χ^2) test for categorical data and the Kruskal-Wallis χ^2 test for quantitative data. P values of ≤ 0.05 were considered significant.

RESULTS

Between July 2006 and June 2009, 2044 stools specimens were collected with a mean rate of 49.9 samples per center and per season. The mean and median ages of the young patients (sex ratio, 1.34) were 13.1 and 10.2 months, respectively (range, 0.2–59.9 months). Most of rotavirus infections occurred in children under 2 years (85.8%).

From the 2044 rotavirus-positive stool samples, 97 (4.7%) could not be amplified by RT-PCR, and 1947 were amplified and characterized further for identification of their G and P genotypes. Of these, 1889 (97.0%) fecal specimens contained only 1 rotavirus strain, and 58 (3.0%) were mixed infections of rotavirus G and P types.

Molecular Characterization of Circulating Rotavirus Strains

G1 rotavirus strains were the most predominant during the course of the study and in most cities with a mean prevalence of 61.7%, followed by G9 strains with 27.4% (see Tables, Supplemental Digital Content 1 and 2, http://links.lww.com/INF/A551 and http://links.lww.com/INF/A552, respectively). The prevalence of other strains was low and varied over time, particularly the G2 strains with a wide range of amplitude (2.0%–13.6%). Mixed infections involved associations of either G1 or G9 with one of the other strains in 96.7%, most often G1 with G9 (58.3%).

Distribution of the genotype combinations by city and year showed heterogeneous circulations of the 2 dominant strains depending on seasons and cities. G9P[8] strains were episodically more prevalent than G1P[8] strains in 7 cities depending on the season, describing than a relative movement southeastward (Fig. 1). In addition, the distribution of P genotype strains showed a clear predominance of P[8] strains with a mean detection rate of 92.9%. The 5 major genotype combinations (G1/G3/G4/G9P[8] and G2P[4]) accounted for 95.5% of the total isolated strains with a progressive increase from season to season from 94.2% to 97.0%. Although most of the strains varied over time, the G9P[8] strains remained stable during the 3 seasons. The detection rate for mixed rotavirus infections was also similar every season (2.9%) (Table 1).

Rotavirus infections occurred throughout the year with a peak between January and March (Fig. 2). The 2006 to 2007 season rotavirus peak occurred 1 to 2 months later than during the subsequent seasons. Differences in peak month were also observed across the cities (data not shown). Analysis of data from the 5 most informative cities showed peak variations for season and location. In the provincial cities, rotavirus infections peaked 2 months later, between February and March, than in Paris where rotavirus infections peaked between January and February.

Thirty-one (1.6%) atypical reassortant rotavirus strains were detected during the surveillance and classified according their 4 main genes (see Table, Supplemental Digital Content 3, http://links.lww.com/INF/A553). Of note, the G12 rotaviruses, mostly associated with human P[8] type, were the most frequent uncommon strains (11 over 31 [35.5%]) and were detected in 6 different cities during the 3 seasons. All G12 strains harbored NSP4 and VP6 genes of human origin. Seven G8 strains (22.6%) were also detected. The G8P[6] strains, detected during each season in Paris, and the G8P[14] strain harbored NSP4 and VP6 genes of bovine origin. Phylogenetic analysis of the VP7 gene showed that the G8P[6] strains belonged to the lineage *d*, whereas the G8P[14] strain belonged to the lineage *a* and shared 99.4% of nucleotides with the A202/06/FR bovine strain (see Fig., Supplemental Digital Content 4, http://links.lww.com/INF/A554). Few G6 strains were detected. Others strains were unusual combination of common G types with common human or potential zoonotic P types such as P[6] (6 [19.4%]) but also P[3], P[9] and P[14].

TABLE 1. Distribution and Detection Rate of G and P Genotype Combinations of Group A Rotavirus Detected in France From 2006 to 2009

	No. Strains Detected During the 3 Consecutive Seasons (%)			Total Strains (%) 2006–2009 N = 1947
	2006–2007 n = 567	2007–2008 n = 736	2008–2009 n = 644	
Common strains	534 (94.2)	701 (95.2)	625 (97.0)	1860 (95.5)
G1P[8]	300 (52.9)	491 (66.7)	362 (56.2)	1153 (59.2)
G2P[4]	70 (12.3)	12 (1.6)	33 (5.1)	115 (5.9)
G3P[8]	16 (2.8)	18 (2.4)	28 (4.3)	62 (3.2)
G4P[8]	5 (0.9)	2 (0.3)	35 (5.4)	42 (2.2)
G9P[8]	143 (25.2)	178 (24.2)	167 (25.9)	488 (25.1)
Unusual strains	13 (2.3)	12 (1.6)	6 (0.9)	31 (1.6)
Reassortment among common human rotavirus strains				
G2P[8]	2 (0.4)	0	0	2 (0.1)
G4P[4]	0	0	1 (0.2)	1 (0.05)
Possible human-animal rotavirus reassortants or zoonotic rotavirus strains				
G1/G9P[6]*	2 (0.4)	0	0	2 (0.1)
G1P[6]	0	0	1 (0.2)	1 (0.05)
G2P[6]	1 (0.2)	1 (0.1)	0	2 (0.1)
G3P[3]	1 (0.2)	0	0	1 (0.05)
G3P[6]	0	0	1 (0.2)	1 (0.05))
G3P[9]	1 (0.2)	0	0	1 (0.05)
G6P[9]	0	1 (0.1)	0	1 (0.05)
G6P[14]	0	1 (0.1)	0	1 (0.05)
G8P[6]	4 (0.7)	1 (0.1)	1 (0.2)	6 (0.3)
G8P[14]	0	0	1 (0.2)	1 (0.05)
G12P[6]	0	1 (0.1)	0	1 (0.05)
G12P[8]	2 (0.4)	7 (1.0)	1 (0.2)	10 (0.6)
Mixed infections†	22 (3.9)	23 (3.1)	13 (2.0)	58 (3.0)

*Theses strains are also included in mixed infections.
†Details on mixed infection strains are reported in Table, Supplemental Digital Content 1, http://links.lww.com/INF/A551.

Clinical Characteristics and Severity of Rotavirus Infections

Clinical records were collected from a group of 630 children who were thereafter hospitalized in pediatric departments. Among them, only 2 infants were vaccinated against rotaviruses. The mean and median ages, and the distribution of rotavirus strains among these infants were similar and representative of the whole cohort of the study. Comparisons of clinical records in relation to genotypes are reported in Table 2. No difference was found in clinical presentation between G genotypes in each comparison group. No difference in severity criteria was found either between each genotype, except that infections with G12P[8] or potential zoonotic strains and mixed infections required a shorter duration of hospitalization ($P = 0.029$). Moreover, no difference in clinical presentation was found between P[4] and P[8] rotavirus infections,

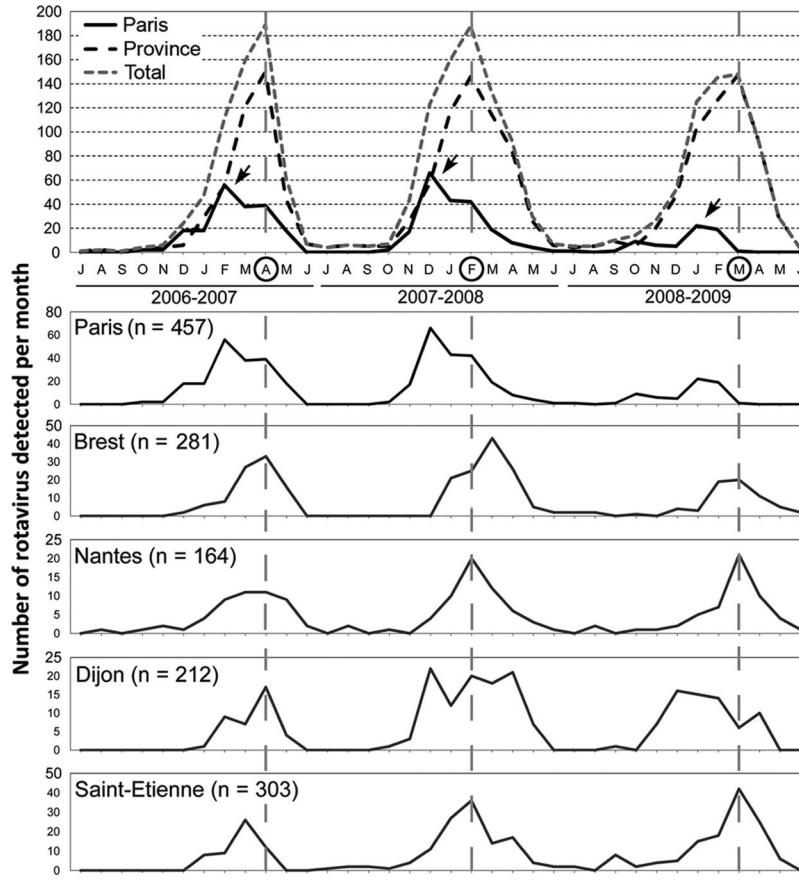

FIGURE 2. Temporal and geographic distribution of rotavirus infections in France from July 2006 to June 2009. Note: the data from the 3 Parisian University Hospitals were brought together for peaks comparison analysis.

except that fever over 38.5°C was significantly more frequent in P[4] infections than P[8] ($P = 0.048$) (data not shown).

Of note, a 12-month-old girl died of a mixed rotavirus infection (G1/G9P[8] strains) upon her arrival at the emergency room as a result of extreme dehydration.

DISCUSSION

In the face of the high disease burden, large studies investigating virologic and clinical features of rotavirus infections are required to optimize vaccination strategies. The French national rotavirus surveillance network monitors more especially rotavirus strains circulating in infants throughout France. The main objective was to provide an extensive and thorough picture of rotavirus infections in infants admitted to pediatric emergency units in the country.

The G1P[8] strains are commonly the most prevalent genotype combination of strains worldwide.[8,9] Uninterrupted rotavirus surveillance for 2 decades in Europe also confirmed that G1P[8] strains were the most common genotype, and that fluctuations of the other genotypes described epidemics peaks during 2 or 3 consecutive seasons. This dominance of G1P[8] strains seems to be due in fact to the emergence of new strains rather than to the reemergence of old strains.[26,27] The sustained circulation of G1 strains is supported by the fact that antigenic G1 variants might appear or disappear alternately under the influence of immune-pressure mechanisms.[28]

Interestingly, G9P[8] strains remained one of the most common types in Europe since their emergence.[29,30] However, unlike in other European countries, where G9P[8] prevalence progressively decreased season after season,[31] in France, G9P[8] strains maintained a relatively stable incidence level of around 25% during the 3 seasons. It has been suggested that the emergence and the persistence of these G9 strains were due to the ability of the phylogenetic lineage III to reassort much more frequently than other strains[8,9], but may also be due to a lack of previous exposure and maternal antibodies in patients.[32] However, it remains unclear whether G9 strains have a selective advantage over other rotaviruses.

Fluctuations of G2P[4] strains, although usually less marked, reflect the normal interseasonal diversity of strains as previously reported[33] driven by the reemergence of VP7 antigenic mutants.[27,33,34] This should be monitored carefully in the French vaccination program for the forthcoming seasons.

The surveillance of various rotavirus strains in the past decade showed a large diversity in rotavirus genotypes, and particularly the establishment of new rotavirus genotypes such as G9. Likewise, the newly detected G12 strains that have already been observed on all continents[31,35–38] are likely to emerge at a higher incidence rates in the future. Indeed, regularly detected since their first detection in 1987, the G12 rotaviruses are currently the most frequent uncommon strains detected in Europe and Australia.[31,37] G12 strains, first detected in France in 2004,[29] in this study were

TABLE 2. Clinical Characteristics According to Rotavirus Genotypes in 630 Hospitalized Diarrheal Children Between 2006 and 2009

	G1P[8] n = 411	G9P[8] n = 141	G2P[4] n = 25	G3P[8] 20	G4P[8] n = 13	G12P[8] n = 6	Zoonotic Strains n = 5	Mixed Infections n = 9	**Total N = 630**	*P**	*P*†	*P*‡
Prevalence	65.2%	22.4%	4.0%	3.2%	2.1%	1.0%	0.8%	1.4%	**100.0%**	—	—	—
Mean age, mo	13.0 ± 11.0	15.3 ± 12.3	17.6 ± 15.8	12.9 ± 7.8	17.1 ± 14.2	11.9 ± 10.0	20.1 ± 20.8	8.1 ± 7.9	**13.8 ± 11.6**	0.020¶	0.129¶	0.096¶
Sex ratio	1.43	1.17	1.50	1.22	0.86	0.20	0.67	2.00	**1.32**	0.279‖	0.684‖	0.432‖
Mean no. diarrhea bouts per day	5.6 ± 4.3	5.9 ± 3.5	4.2 ± 2.6	7.2 ± 4.7	4.4 ± 4.0	4.4 ± 3.0	3.8 ± 1.9	2.4 ± 2.3	**5.6 ± 4.1**	0.141¶	0.231¶	0.073¶
Mean no. vomiting bouts per day	3.1 ± 3.5	3.7 ± 3.9	2.4 ± 1.7	2.4 ± 2.2	3.3 ± 2.2	2.0 ± 2.7	2.7 ± 3.8	1.3 ± 1.0	**3.1 ± 3.5**	0.111‖	0.544‖	0.584‖
Blood in stool	5 (1.4%)	1 (0.8%)	1 (5.9%)	0	0	0	0	0	**7 (1.1%)**	0.616‖	0.614‖	0.908‖
Fever >38.5°C	199 (48.3%)	61 (43.0%)	16 (62.5%)	8 (38.9%)	5 (37.5%)	2 (33.3%)	3 (60.0%)	3 (28.6%)	**297 (47.0%)**	0.309‖	0.261‖	0.583‖
Temperature, °C	38.2 ± 1.0	38.0 ± 1.0	38.5 ± 1.0	38.0 ± 0.9	37.6 ± 1.0	37.7 ± 1.1	38.3 ± 11	37.8 ± 1.0	**38.2 ± 1.0**	0.129¶	0.072¶	0.197¶
Dehydration	275 (66.9%)	101 (71.6%)	17 (68.0%)	16 (80.0%)	10 (76.9%)	2 (33.3%)	2 (40.0%)	6 (66.7%)	**429 (68.1%)**	0.351‖	0.794‖	0.167‖
Dehydration degree, %	6.6 ± 2.8	6.4 ± 2.7	6.1 ± 2.6	6.0 ± 2.7	6.5 ± 1.2	6.5 ± 2.1	4.0 ± 2.8	4.4 ± 1.5	**6.5 ± 2.7**	0.359¶	0.476¶	0.317¶
Intravenous rehydration	278 (67.6%)	105 (74.5%)	17 (68.0%)	15 (75.0%)	9 (69.2%)	3 (50.0%)	5 (100.0%)	6 (66.7%)	**438 (69.5%)**	0.129‖	0.667‖	0.548‖
Oral rehydration	119 (29.0%)	39 (27.7%)	4 (16.0%)	2 (10.0%)	3 (23.1%)	1 (16.7%)	0	5 (55.6%)	**173 (27.5%)**	0.769‖	0.248‖	0.134‖
Intensive care	8 (1.9%)	1 (0.7%)	0	0	0	0	0	0	**9 (1.4%)**	0.316‖	—	—
Hospitalization duration, d	3.1 ± 2.6	3.1 ± 1.9	3.0 ± 1.6	3.2 ± 1.5	3.2 ± 1.5	1.2 ± 0.4	2.2 ± 0.8	2.1 ± 1.1	**3.1 ± 2.3**	0.215¶	0.648¶	0.029¶

*Comparison between G1P[8] and G9P[8] groups.
†Comparison within the 5 main genotype groups.
‡Comparison within all genotype groups.
¶Using Kruskal-Wallis χ^2 test.
‖Using Pearson χ^2 test.
Statistically significant if $P < 0.05$.

associated with 2 different P types, P[6] and P[8], confirming a high level of adaptation to humans and ability to reassort, all the more so as they have also been found in combination with the P[4], P[9], and P[14] genotypes.[35,39,40] G12P[4] and G12P[8] strains are more likely to be the result of reassortment between human and animal strains, whereas rotavirus with other combinations may have emerged from zoonotic infections. This abundance of mixtures suggests environmental or waterborne transmissions that require further investigations to evaluate their potential emergence as future predominant strains, their fitness apparently being actually insufficient to allow easy transmission between humans.

The incidence of infections with viruses of possible zoonotic origin was regularly observed during the 3 seasons, notably G6 and G8 strains, which harbored NSP4 and VP6 genes from animals, particularly from bovine hosts, and might result in natural reassortment during animal-human transmissions or human mixed rotavirus infections from environmental reservoirs.[41,42] Interestingly, the close similarity of the reported G8P[6] strains and their constant circulation within the Parisian population probably indicates transmission of human-adapted strains. Conversely, the VP7 gene of the R3265 strain was similar to a previous strain detected in a diarrheal calf in Dijon, France, in 2006 thus showing animal-adapted rotavirus circulation.

Furthermore, 6 of the uncommon strains had P types of feline, canine, or lapine origin (P[3], P[9], and P[14]) suggesting that they might be introduced in humans by infected pets, whose role in transmission remains to be determined, or during contact with a mixture of human and animals strains from soiled environment or water. These observations demonstrate the interspecies transmission of group A rotaviruses, notably from animals living closely with humans resulting in the introduction of new rotavirus genotypes in humans. Further molecular analyses should clarify the animal origins of these strains, indicate their ability to adapt to humans and help to determine the likelihood of emergence to higher incidence rates in the human population.

In Europe, rotavirus infections occur especially during winter epidemics with a geographic gradient of increasing incidence from south-west toward the north, and the intensity of which varies according to the seasons and the country.[43,44] In France, the winter rotavirus epidemics happened 1 to 2 months earlier in Paris than in the provincial cities. A similar observation has been reported also for influenza in France (data from the National Reference Center for influenza, Pasteur Institute, Paris, France). The high density of populations that facilitates high contact frequency between individuals in the large cities might also play a substantial role in the epidemic peak shifting. In addition, seasonal disparities remain in the geographic distribution of various G genotypes between cities, which could be due to the preferential local circulation of specific rotavirus strains, many rotavirus infections being acquired in child-care centers.

Analysis of the clinical records of a group of 630 children hospitalized for rotavirus-induced acute gastroenteritis showed no difference in clinical manifestations or severity in relation to rotavirus genotypes. Only few studies have previously addressed this issue. Commonly, differences in severity may be explained by variations in virulence between rotavirus strains and by the immune status of the population toward a serotype which is partially dependent upon the infecting strain.[45]

Some studies have been generally inconclusive due to the small number of rotavirus episodes studied or because the severity of dehydration was limited in range. Only 2 studies found that genotypes G2 or G2 and G3 were associated with more-severe gastroenteritis than were G1 or G4,[46,47] and 2 others showed that G9 infections were more severe than were G1 or other genotype infections.[48,49] G9 infections were found to be more severe in a Brazilian vaccine efficacy trial,[48] but these data were biased due to an artificial increase in G9 prevalence combined with an alteration in the severity score caused by the inclusion of patients presenting 2 successive rotavirus infections. A British study also reported higher severity in G9 infections among hospitalized patients, but

with G9P[6] strains, which are rarely detected in France.[49] Conversely, no difference in severity between G1 and G9 infections were found in 2 European studies,[50,51] nor after the re-emergence of the G9 genotype after a long absence in the United States.[52] Of note, disease from infections with G12P[8] and potential zoonotic strains required shorter duration of hospitalization. This might be associated with incomplete adaptation to humans.

Thus, our data suggest that G or P types as well as their combination are not the only viral factor responsible for gastroenteritis severity. Other viral factors, such as the NSP4 enterotoxin and NSP1, and the immune status after previous rotavirus infections may influence the intensity and the severity of the clinical symptoms. The data also strongly suggest that rotaviruses of all genotypes may cause acute infections requiring emergency medical care in infants, and reinforce the need of vaccines that ensure effective protection against all rotavirus infections.

In summary, establishing genotypes of rotavirus strains cocirculating in the human population remains a key issue in understanding of the mechanisms by which rotavirus strains emerge or are maintained in the population. The relative stability of rotavirus genotypes currently cocirculating in France may ensure vaccine effectiveness in the short and medium term. However, the likely emergence of G12 and G8 strains should be monitored during ongoing and future vaccination programs, especially as all genotypes can cause severe infections. The surveillance of rotavirus infections should continue to monitor the emergence of new reassortants that may not respond to current rotavirus vaccines. Large scale rotavirus survey will help to create an appropriate vaccine strategy against rotavirus disease.

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