Clinical severity and molecular characteristics of circulating and emerging rotaviruses in young children attending hospital emergency departments in France

A. Rougemont, J. Kaplon, C. Fremy, S. Aho, P. Pothier, M.-C. Legrand-Guillien, A. Minoui-Tran, C. Payan, A. Vabret, L. Mendes-Martins, et al.

Title: Clinical severity and molecular characteristics of circulating and emerging rotaviruses in young children attending hospital emergency departments in France

Authors: Alexis de ROUGEMONT* [1,2], Jérôme KAPLON [1], Céline FREMY [1], Serge AHO [3], Pierre POTHIER [1,2] and the French National Rotavirus Network.

The French National Rotavirus Network investigators: Marie-Christine LEGRAND-GUILLIEN [4], Adissa MINOUI-TRAN [4], Christopher PAYAN [4], Astrid VABRET [5], Lucile MENDES-MARTINS [6], Mondher CHOUCHANE [7], Raphaëlle MAUDINAS [7], Frédéric HUET [2,7], François DUBOS [8], Didier HOBER [8], Mouna LAZREK [8], Christelle BOUQUIGNAUD [9], Anne DECOSTER [9], Sophie ALAIN [10], Jeanne LANGUEPIN [10], Yves GILLET [11], Bruno LINA [11], Yahia MEKKI [11], Florence MORFIN-SHERPA [11], Aurélie GUIGON [12], Jérôme GUINARD [12], Vincent FOULONGNE [13], Michel RODIERE [13], Véronique AVETTAND-FENOEL [14], Stéphane BONACORSI [14], Antoine GARBARG-CHENON [14], Dominique GENDREL [14], Pierre LEBON [14], Mathie LORROT [14], Patricia MARIANI [14], Jean-François MERITET [14], Aurélie SCHNURINGER [14], Gérard AGIUS [15], Agnès BEBY-DEFAUX [15], Denis ORIOT [15], Ronald COLIMON [16], Gisèle LAGATHU [16], Olivier MORY [17], Sylvie PILLET [17], Bruno POZZETTO [17], Jean-Louis STEPHAN [17].

* **Corresponding author:** Dr. Alexis de Rougemont, Centre National de Référence des virus entériques, Laboratoire de Virologie, CHU de Dijon, 2 rue Angélique Ducoudray, BP 37013, F-21070 Dijon Cedex, France; phone number: +33 (0) 380293170; fax number: +33 (0) 380293280; email: alexis.de-rougemont@u-bourgogne.fr.

[1] Centre National de Référence des virus entériques, Laboratoire de Virologie, CHU de Dijon, France

[2] UFR des Sciences de Santé, Université de Bourgogne, Dijon, France

[3] Service d'Hygiène Hospitalière, Centre Hospitalier Universitaire de Dijon, France

[4] Centre Hospitalier Universitaire de Brest, France

[5] Centre Hospitalier Universitaire de Caen, France

[6] Centre Hospitalier de Charleville-Mézières, France

[7] Centre Hospitalier Universitaire de Dijon, France

[8] Centre Hospitalier Régional Universitaire de Lille, France
[9] Groupement des Hôpitaux de l'Institut Catholique de Lille, France
[10] Centre Hospitalier Universitaire de Limoges, France
[11] Hospices Civils de Lyon, France
[12] Centre Hospitalier Universitaire d'Orléans, France
[13] Centre Hospitalier Universitaire de Montpellier, France
[14] Assistance Publique Hôpitaux de Paris, France
[15] Centre Hospitalier Universitaire de Poitiers, France
[16] Centre Hospitalier Universitaire de Rennes, France
[17] Centre Hospitalier Universitaire de Saint-Etienne, France

ABSTRACT

Objectives

Group A rotavirus (RVA) is the leading cause of acute gastroenteritis in young children worldwide. A prospective surveillance network has been set up to investigate the virological and clinical features of RVA infections and to detect the emergence of potentially epidemic strains in France.

Methods

From 2009 to 2014, RVA-positive stool samples were collected from 4800 children under 5 years old attending the paediatric emergency units of 16 large hospitals. Rotaviruses were then genotyped by RT-PCR with regard to their outer capsid proteins VP4 and VP7.

Results

Genotyping of 4708 RVA showed that G1P[8] strains (62.2%) were predominant. The incidence of G9P[8] (11.5%), G3P[8] (10.4%) and G2P[4] (6.6%) strains varied considerably, whilst G4P[8] (2.7%) strains were circulating mostly locally. Of note, G12P[8] (1.6%) strains emerged during the seasons 2011-12 and 2012-13 with 4.1% and 3.0% of prevalence, respectively. Overall, 40 possible zoonotic reassortants, such as G6 (33.3%) and G8 (15.4%) strains, were detected, and were mostly associated with P[6] (67.5%). Analysis of clinical records of 624 hospitalized children and severity scores from 282 of them showed no difference in clinical manifestations or severity in relation to the genotype.

Conclusions

The relative stability of RVA genotypes currently co-circulating and the large predominance of P[8] type strains may ensure vaccine effectiveness in France. The surveillance will continue to monitor the emergence of new reassortants that might not respond to current vaccines, all the more so as all genotypes can cause severe infections in infants.

INTRODUCTION

Group A rotavirus (RVA) is the leading cause of acute gastroenteritis (AGE) in young children worldwide, and is estimated to cause around 453,000 deaths every year in children under 5 years old, mostly in developing countries **(1)**. Although fast and appropriate care has considerably reduced mortality in industrialised countries, rotaviruses are still responsible for considerable morbidity in infants and generate significant health costs.

RVAs belong to the *Reoviridae* family and possess a genome composed of 11 dsRNA genomic segments encoding six structural (VPs) and five/six non-structural (NSPs) proteins. On the basis of the outer capsid proteins VP7 and spikes VP4, RVA can be classified into G and P genotypes, respectively. Nucleotide differences in these two genes currently allow the classification of RVA in 27 G- and 37 P-genotypes, among which 12 G and 15 P are associated with infections in humans, thus showing a considerable diversity of strains **(2, 3)** . Both viral outer layer proteins elicit the production of neutralizing antibodies in the host, but no precise G or P type clearly correlates with the severity of the disease. The five RVA genotype combinations G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] are responsible for approximately 90% of RVA infections in children **(4)**. Of note, uncommon G types such as G5, G8, G10 and G12 have emerged in various areas of the world, notably in tropical regions **(5, 6)**.

Local data on the current burden of rotavirus disease, including both virological and clinical aspects, are important for decision-making and optimization regarding immunization strategies **(7)**. Hence, knowledge of the molecular epidemiology and antigenic diversity of co-circulating rotaviruses is necessary to ensure the suitability and the efficacy of vaccines. Indeed, RVA 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 **(8)**. Programs to monitor rotavirus antigenic drifts that might be caused by

specific immunologic pressures and to monitor potential reassortments between human or human and animal strains have to be carefully developed.

In France, rotavirus infections occur mostly during the winter and spring seasons. Although deaths remain exceptional, RVA are responsible for about 300,000 acute diarrhoea episodes, half of which are severe, 140,000 consultations and 18,000 hospitalisations each year **(9)**. In spite of the introduction of RVA vaccines in 2006, coverage remains particularly low in France and was estimated at around 8.9% in French infants under 12 months old in 2011 **(10)**. Since the establishment of the French rotavirus surveillance network by the National Reference Centre for Enteric Viruses (Dijon, France), it has become possible to assess the nationwide circulation of RVA 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 attending paediatric emergency units during the rotavirus vaccine era in France. Special attention was paid to the detection of uncommon strains and emerging reassortants.

METHODS

After its approval by the local ethical committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Bourgogne, Dijon, France, on the 24th of November 2005), the surveillance study was conducted during five consecutive seasons from July 2009 to June 2014, and involved 4800 children under 5 years old suffering from rotavirus-induced acute gastroenteritis and attending the paediatric emergency units of 16 French Hospitals in 13 regions, including Paris.

Acute gastroenteritis was defined by at least three soft or liquid stools or three bouts of vomiting in 24h. Children presenting with chronic diarrhoea, immune deficiency, inflammatory disease of the digestive tract, or nosocomial infections were excluded. Clinical data, including personal identification and clinical symptoms, were collected from children presenting to paediatrics departments. Informed consent was obtained for all subjects. Disease severity was calculated using the Vesikari scale, a 0-20 point numerical score which assesses the clinical severity of rotavirus infections (where higher scores indicate greater severity), as previously described **(11)**. An episode of gastroenteritis with a score ≥11 is considered a severe episode.

The stool samples were routinely screened for RVA using mainly immunochromatographic (ICG) tests or enzyme immuno-assays (EIA). All rotavirus-positive samples were stored at -20°C until genotyping by the National Reference Centre for Enteric Viruses, University Hospital of Dijon, France. The rotavirus strains were genotyped using RT-PCR according to the EuroRotaNet methods (www.eurorota.net/docs.php) and using the Qiagen OneStep RT-PCR kit (Qiagen, Hilden, Germany). The VP7, VP4, VP6 and NSP4 PCR products from strains of interest were sequenced with the same primers as for amplifications. All the sequencing reactions were performed using the ABI® PRISM® Terminator Cycle Sequencing Kit on a 3130XL DNA Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The

nucleotide sequences were edited and genotyped using BioNumerics software (Applied Maths NV, Sint-Martens-Latem, Belgium) and a selection of sequences from rotavirus reference strains available from the GenBank database. Phylogenetic analysis was performed using MEGA6 software **(12)**. After sequence alignment using the MUSCLE programme **(13)**, phylogenetic tree was inferred using the Maximum Likelihood method based on the Tamura 3-parameter model, which was the best-fit DNA substitution model for the nucleotide dataset submitted. Bootstrap values were calculated for 1000 replicates. The nucleotide sequences of representative G12 strains from this study have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers: KU291317 to KU291349.

Statistical analyses were performed using STATA® v11.0 software from StataCorp (College Station, TX, USA). Comparisons of clinical data and severity scores between G1 and G12 RVA, and between all RVA genotypes were performed using the Fisher-exact test for categorical data and the Kruskal-Wallis test for quantitative data. Fractional polynomials were used to model the relationship between children's ages and severity scores using a linear regression model including a non-zero intercept. P values of ≤ 0.05 were considered significant.

RESULTS

From July 2009 to June 2014, 4800 stools specimens were collected with a mean rate of 73.8 samples per centre and per season (i.e. a 12-month period that begins in July and ends in June of the year after). The mean and median ages of the young patients were 13.7 and 10.5 months, respectively (range, 0.2 to 59.9 months) and the male/female ratio was 1.34. Most rotavirus infections occurred in children under 2 years old (83.9%). From the 4800 rotavirus-positive stool samples, 92 (1.9%) could not be genotyped by RT-PCR, and 4708 were successfully genotyped. Of these, 4578 (97.2%) faecal specimens contained only one RVA strain, 64 (1.4%) were mixed infections of G and P types, and 66 (1.4%) were partially genotyped.

RVA infections occurred in France throughout the year with a peak between January and April (**Figure 1A**). However, in most of the provincial cities, RVA infections were peaking 2 months later (i.e between February and March) than in the largest cities in our network (i.e. between January and February in Paris and Lyon) (**Figure 1B**).

Molecular distribution of circulating rotavirus strains

G1 strains were the most predominant during the course of the study and in most cities with a mean prevalence of 63.5% [53.5-73.8]. The four other major G genotypes (G2, G3, G4 and G9) accounted for 34.0% [17.9-44.7] of infections with wide individual amplitude according to the season (**Tables S1** and **S2**). Of note, G12 strains accounted for 1.7% [0.0-3.3]. Mixed infections involved associations of G1 strains with one of the other major G genotypes in 75.5% [57.1-100.0]. In addition, the distribution of P genotypes showed a clear predominance of P[8] strains with a mean detection rate of 91.2% [76.6-97.9] followed by P[4] strains with 8.0% [1.4-22.1]. The study of the distribution of G genotype prevalence per month showed that G2 and G9 RVA infections were more frequently detected during the first part of the

175 epidemic season (i.e. from September to January) whereas G3, G4, G12 and to a lesser extent
176 G1 RVA infections were more frequent during the second part and at the peak of the epidemic
177 (from January to June) (**Figures S1A and S1B**). Moreover, no predominant RVA genotype
178 was detected according to the age group.
179
180 Distribution of the genotype combinations showed a clear predominance of G1P[8] strains
181 (62.2% [54.3-73.1]) associated with heterogeneous circulation of secondary major strains, i.e.
182 G9P[8] (11.5% [7.3-22.0]), G3P[8] (10.4% [1.5-19.3]), G2P[4] strains (6.6% [1.1-17.5]) and
183 G4P[8] (2.7% [0.8-7.3]), depending on seasons (**Table 1**). The five major genotype
184 combinations accounted for 93.3% [92.3-96.2] of strains, showing a relatively stable detection
185 rate from season to season. Of note, G2P[4] strain circulation rapidly decreased from 17.5%
186 during the 2009-2010 season to reach 1.1% during the last season, whilst G9P[8] strains,
187 which remained stable at 8.9% [6.3-11.5] during the first four seasons, increased to 22.0%
188 during the 2013-2014 season. The circulation of G3P[8] strains increased significantly during
189 the last three seasons to reach a mean detection rate of 15.5% [13.2-19.3] **(Figure S2)**.
190 Combinations of major human RVA genotypes (i.e. P[4] with either G1, G3, G4 and G9; and
191 P[8] with G2) were regularly detected during the study with a mean prevalence of 1.7% [0.6-
192 3.2] but were decreasing over time **(Table 1)**.
193
194 **Emergence of G12P[8] rotavirus strains**
195 During the study period, 78 G12P[8] strains were detected with a mean prevalence of 1.6%
196 [0.0-4.1], of which 68 were detected during the 2011-12 and 2012-13 seasons with 4.1%
197 (n=35) and 3.0% (n=33) of prevalence, respectively. At the regional level, G12P[8] strains
198 emerged in 2011-2012 at various rates in all regions except Dijon and Charleville-Mézières
199 **(Figure S3)**. They were still detected during the subsequent season in only five cities (Brest,

Lille, Paris, Poitiers and Saint-Etienne). VP7 phylogenetic analysis showed that all these strains belonged to lineage III and segregated into two relatively distinct main clusters that differed from those of the older G12P[8] strains circulating in France between 2005 and 2009, albeit, only three strains detected in 2014 (R8391, R8422 and R8964) were more closely related to these older G12P[8] strains (**Figure S4**). All G12P[8] strains harboured VP6 genes of human origin (genotype I1). However, among these, two strains harboured NSP4 genes of animal origin (genotype E2), whilst all of the others were of human origin (genotype E1).

Human and animal reassortant rotaviruses

Forty (0.8%) atypical RVA strains, of which 27 (67.5%) were P[6] strains, were detected during the surveillance and further characterized according to their VP6 and NSP4 coding genes (**Table S3**). Among these, 20 (51.3%) strains bore genotypes commonly found in cattle: 13 (33.3%) G6, six (15.4%) G8 and one (2.6%) G10 strains. Among the G8 strains, three were combined with a major human P type: two with P[8] and one with P[4] in three different locations and seasons. The single G10 strain was combined with P[8] and harboured VP6 and NSP4 genes of human origin. Other strains were unusual combinations of common G types with common human or potential zoonotic P types such as P[3], P[6], P[9] and P[14].

Clinical characteristics and severity of rotavirus infections

Clinical records could be collected from 624 children who were thereafter hospitalized in paediatrics departments. Comparisons of clinical records in relation to genotypes are reported in **Table 2**. No difference was found between RVA genotypes for the clinical presentation. None of the patients had been vaccinated. No deaths were reported during the study period. Vesikari severity scores could be calculated for 282 children from the same cohort. In all, 148 (51.7%) infants, most of whom were more than 6 months old (91.9%), hospitalized with

rotavirus gastroenteritis were classified as severe according to the Vesikari scale (i.e. a score ≥11 points). Severity was significantly lower in children aged 0-6 months with a mean score of 8.9 ±2.7 (P<0.0001) than in the three other age groups. A linear regression model using fractional polynomials was used to study this relationship, i.e. between the children's age in months and the severity score, and no linear relation was found as shown in **Figure S5**. The mean Vesikari severity score of the 282 rotavirus infections was calculated at 10.30 ±3.3 where G1P[8] score was 10.24 ±3.9 and G12P[8] score was 10.13 ±2.8. No difference in Vesikari severity score was found between G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] (P=0.604), between G1P[8] and G12P[8] (P=0.813) or between P[4] (9.93 ±2.9) and P[8] (10.38 ±3.4) RVA infections (P=0.386).

DISCUSSION

During this 5-year study, we have showed that G12P[8] RVA strains have slightly emerged in France during two consecutive seasons, and confirmed that RVA infection severity is not related to genotype.

Although rotavirus infections in Europe have been reported to occur with a geographical gradient of increasing incidence from the south-west towards the north **(14)**, at the country level, they seem to spread more according to centrifugal progression from areas where the population density is the highest towards the lowest. In France, the winter rotavirus epidemics occurred globally 2 months earlier in large cities than in smaller provincial cities, as previously reported **(15)**. Indeed, high population densities in the large cities facilitate contact between individuals and may thus play a substantial role in shifting the epidemic peak.

During the study, the circulation of the G1P[8] strain in France was overall predominant and similar to that in the rest of the world despite the unexpected transient emergence of new major genotypes. This predominance may be due to the emergence of new antigenic G1 variants appearing or disappearing alternately under the influence of natural immune-pressure mechanisms **(16)**.

Following their emergence in France, G9P[8] strains maintained a relatively high incidence level **(15)**, before strongly decreasing during the current study period. Interestingly, this decline occurred much later in France than in other European countries, where, after emergence, the prevalence of G9P[8] strains progressively decreased season after season **(17)**. However, during the last season in France, high G9P[8] prevalence was observed again. To date, there is no evidence of any particular environmental or immune factors that could explain the long circulation of G9 strains or their renewed circulation in French infants.

During the study, G2P[4] prevalence was markedly high during the 2009-2010 season, whilst it was negligible in the rest of the study period. These fluctuations reflect here the normal

interseasonal diversity of strains driven by the reemergence of VP7 antigenic mutants, or potentially due to reassortment among co-circulating strains or an introduction of new lineages of G2 strains or antigen combinations **(18)**.

While the detection rate for G4P[8] was relatively negligible during the study, the circulation of G3P[8] strains increased and then stabilized at around 14%. This increased frequency was supposedly at the expense of the decrease in the frequencies of G1P[8] and G9P[8] **(19, 20)**, but could just be related to the re-emergence of G3 antigenic mutants.

G12 strains, first detected in France in 2004, were the most frequent among the uncommon strains detected in Europe and Australia **(17, 21)**. Recently, G12P[8] have emerged during the 2011-12 season in 11 of the 13 monitored regions but were only persistent in 5 regions during the subsequent season. This particularly mild emergence may be probably explained by both a herd immunity against P[8] types and an acquired cross-reactive immunity of the population. Indeed, it differs from the intense emergence reported in the North of Spain during the 2010-2011 season **(22)**, although it could be related the more so as the two countries share the same borders and the Spanish G12 emergence preceded only by 1 season the French one. Phylogenetically, these G12 strains belong to the lineage III but diverge from the older strains previously detected, suggesting that an evolution of VP7 antigens or an introduction of new strains in the country have occurred.

Infections with viruses of possible zoonotic origin was regularly observed during the 5 seasons, notably G6 and G8 strains associated with P[6] or P[14], which harboured NSP4 and VP6 genes from porcine and bovine hosts, and might have resulted from natural reassortment during animal-human transmissions or mixed human rotavirus infections from environmental reservoirs **(23, 24)**. These observations demonstrate the interspecies transmission of RVAs, notably from animals living in close contact with humans, resulting in the introduction of new rotavirus genotypes in humans. Interestingly, two G8P[8] and one G8P[4] strains were

detected in three different places showing that some G8 strains might initiate their adaption to humans, as previously observed for G4, G9 and G12 strains. While the G8P[4] strain was associated with animal VP6 and NSP4 and was seemingly related to African strains **(25)**, both G8P[8] strains harboured human VP6 and NSP4 genes, thus showing active recombination with human P[8] strains, as previously reported **(26)**. These human G8P[8] strains require further investigations to evaluate their potential emergence; their fitness is currently insufficient to allow easy transmission to humans.

Analysis of the clinical records showed no difference in clinical manifestations or severity in relation to genotype. These results are consistent with our previous findings **(15, 27)**. More than half of the rotavirus infections in these children were considered severe, which is consistent with previous studies **(28, 29)**. RVA infections in children under 6 months old were, however, less severe than those in any other age group, probably thanks to the protection provided by maternal antibodies. Commonly, differences in severity may be explained by variations in virulence between strains and by the immune status of the population towards a serotype which is partially dependent upon the infecting strain. However, globally, most studies on rotavirus infection severity have been either inconclusive or contradictory **(27, 30-32)**. Nevertheless, both VP7 and VP4 antigens induce antibodies, and most of the genotypes compared had the same P[8] type, thus abrogating the differences between G types. As the severity of the disease does not depend on age but on the infecting virus and previous exposure **(33)**, severe infections may be found mostly during primary infection or exposure to new antigens, whilst less severe infections occur after second exposure, during which heterotypic protection may reduce the symptoms and shorten the infection. These data therefore suggest that VP4 and VP7 antigens are not the only factors responsible for the severity of rotavirus infections. Other strain-specific viral factors and

immune status, whether naive or after previous infections, influence the intensity and the severity of the clinical symptoms. The data also strongly suggest that all RVA genotypes may cause acute gastroenteritis, as their G and P types cannot be dissociated, and thus reinforce the need for vaccines that ensure effective protection, whether directly or by cross-reaction, against all rotavirus infections.

Today, rotavirus vaccination is still too low in France to have any impact on annual epidemics other than locally. The relative stability of RVA genotypes currently co-circulating and the large predominance of P[8] type strains may ensure vaccine effectiveness, and, given the good outcomes of vaccination in countries with sufficient coverage **(34)**, extensive vaccination should be encouraged in France. The surveillance of rotavirus infections during ongoing and future vaccination programs will ensure the detection of emergent new reassortants and will help the health authorities to optimize appropriate vaccination strategies against rotavirus diseases, all the more so as all genotypes can cause severe infections in infants.

TRANSPARENCY DECLARATION

The authors declare no competing financial interests in the conduct of the study. This work received funding from the EuroRotaNet project and SPMSD through a non-restrictive collaborative grant.

REFERENCES

1. Tate JE, Burton AH, Boschi-Pinto C, Steele AD, Duque J, Parashar UD, et al. 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis. Lancet Infect Dis. 2012;12(2):136-41.
2. Trojnar E, Sachsenroder J, Twardziok S, Reetz J, Otto PH, Johne R. Identification of an avian group A rotavirus containing a novel VP4 gene with a close relationship to those of mammalian rotaviruses. J Gen Virol. 2013;94(Pt 1):136-42.
3. Matthijnssens J, De Grazia S, Piessens J, Heylen E, Zeller M, Giammanco GM, et al. Multiple reassortment and interspecies transmission events contribute to the diversity of feline, canine and feline/canine-like human group A rotavirus strains. Infect Genet Evol. 2011;11(6):1396-406.
4. Iturriza-Gomara M, Dallman T, Banyai K, Bottiger B, Buesa J, Diedrich S, et al. Rotavirus genotypes co-circulating in Europe between 2006 and 2009 as determined by EuroRotaNet, a pan-European collaborative strain surveillance network. Epidemiol Infect. 2011;139(6):895-909.
5. Santos N, Hoshino Y. Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. Rev Med Virol. 2005;15(1):29-56.
6. Todd S, Page NA, Duncan Steele A, Peenze I, Cunliffe NA. Rotavirus strain types circulating in Africa: Review of studies published during 1997-2006. J Infect Dis. 2010;202 Suppl:S34-42.
7. Matthijnssens J, Bilcke J, Ciarlet M, Martella V, Banyai K, Rahman M, et al. Rotavirus disease and vaccination: impact on genotype diversity. Future Microbiol. 2009;4(10):1303-16.
8. Ciarlet M, Hoshino Y, Liprandi F. Single point mutations may affect the serotype reactivity of serotype G11 porcine rotavirus strains: a widening spectrum? J Virol. 1997;71(11):8213-20.
9. Melliez H, Boelle PY, Baron S, Mouton Y, Yazdanpanah Y. [Morbidity and cost of rotavirus infections in France]. Med Mal Infect. 2005;35(10):492-9.
10. Martinot A, Cohen R, Denis F, Gaudelus J, Lery T, Lepetit H, et al. [Assessing early childhood vaccination coverage in France after 2013 vaccination schedule implementation]. Arch Pediatr. 2014;21(12):1389-90.
11. Ruuska T, Vesikari T. Rotavirus disease in Finnish children: use of numerical scores for clinical severity of diarrhoeal episodes. Scand J Infect Dis. 1990;22(3):259-67.
12. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30(12):2725-9.
13. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32(5):1792-7.

14. Koopmans M, Brown D. Seasonality and diversity of Group A rotaviruses in Europe. Acta Paediatr Suppl. 1999;88(426):14-9.

15. de Rougemont A, Kaplon J, Pillet S, Mory O, Gagneur A, Minoui-Tran A, et al. Molecular and clinical characterization of rotavirus from diarrheal infants admitted to pediatric emergency units in france. Pediatr Infect Dis J. 2011;30(2):118-24.

16. Arista S, Giammanco GM, De Grazia S, Ramirez S, Lo Biundo C, Colomba C, et al. Heterogeneity and temporal dynamics of evolution of G1 human rotaviruses in a settled population. J Virol. 2006;80(21):10724-33.

17. Iturriza-Gomara M, Dallman T, Banyai K, Bottiger B, Buesa J, Diedrich S, et al. Rotavirus surveillance in europe, 2005-2008: web-enabled reporting and real-time analysis of genotyping and epidemiological data. J Infect Dis. 2009;200 Suppl 1:S215-21.

18. Wu FT, Banyai K, Jiang B, Wu CY, Chen HC, Feher E, et al. Molecular epidemiology of human G2P[4] rotaviruses in Taiwan, 2004-2011. Infect Genet Evol. 2014;28:530-6.

19. Stupka JA, Degiuseppe JI, Parra GI, Argentinean National Rotavirus Surveillance N. Increased frequency of rotavirus G3P[8] and G12P[8] in Argentina during 2008-2009: whole-genome characterization of emerging G12P[8] strains. J Clin Virol. 2012;54(2):162-7.

20. Mitui MT, Chan PK, Nelson EA, Leung TF, Nishizono A, Ahmed K. Co-dominance of G1 and emerging G3 rotaviruses in Hong Kong: a three-year surveillance in three major hospitals. J Clin Virol. 2011;50(4):325-33.

21. Kirkwood CD, Boniface K, Bogdanovic-Sakran N, Masendycz P, Barnes GL, Bishop RF. Rotavirus strain surveillance--an Australian perspective of strains causing disease in hospitalised children from 1997 to 2007. Vaccine. 2009;27 Suppl 5:F102-7.

22. Cilla G, Montes M, Gomariz M, Alkorta M, Iturzaeta A, Perez-Yarza EG, et al. Rotavirus genotypes in children in the Basque Country (North of Spain): rapid and intense emergence of the G12[P8] genotype. Epidemiol Infect. 2013;141(4):868-74.

23. Rahman M, De Leener K, Goegebuer T, Wollants E, Van der Donck I, Van Hoovels L, et al. Genetic characterization of a novel, naturally occurring recombinant human G6P[6] rotavirus. J Clin Microbiol. 2003;41(5):2088-95.

24. Cunliffe NA, Gentsch JR, Kirkwood CD, Gondwe JS, Dove W, Nakagomi O, et al. Molecular and serologic characterization of novel serotype G8 human rotavirus strains detected in Blantyre, Malawi. Virology. 2000;274(2):309-20.

25. Nyaga MM, Stucker KM, Esona MD, Jere KC, Mwinyi B, Shonhai A, et al. Whole-genome analyses of DS-1-like human G2P[4] and G8P[4] rotavirus strains from Eastern, Western and Southern Africa. Virus Genes. 2014;49(2):196-207.

26. Delogu R, Lo Presti A, Ruggeri FM, Cella E, Giovanetti M, Ciccozzi M, et al. Full-genome characterization of a G8P[8] rotavirus that emerged among children with diarrhea in Croatia in 2006. J Clin Microbiol. 2013;51(5):1583-8.

27. Aupiais C, de Rougemont A, Menager C, Vallet C, Brasme JF, Kaplon J, et al. Severity of acute gastroenteritis in infants infected by G1 or G9 rotaviruses. J Clin Virol. 2009;46(3):282-5.

28. Matthijnssens J, Zeller M, Heylen E, De Coster S, Vercauteren J, Braeckman T, et al. Higher proportion of G2P[4] rotaviruses in vaccinated hospitalized cases compared with unvaccinated hospitalized cases, despite high vaccine effectiveness against heterotypic G2P[4] rotaviruses. Clin Microbiol Infect. 2014;20(10):O702-10.

29. Lewis KD, Dallas MJ, Victor JC, Ciarlet M, Mast TC, Ji M, et al. Comparison of two clinical severity scoring systems in two multi-center, developing country rotavirus vaccine trials in Africa and Asia. Vaccine. 2012;30 Suppl 1:A159-66.

30. Cascio A, Vizzi E, Alaimo C, Arista S. Rotavirus gastroenteritis in Italian children: can severity of symptoms be related to the infecting virus? Clin Infect Dis. 2001;32(8):1126-32.

31. Linhares AC, Verstraeten T, Wolleswinkel-van den Bosch J, Clemens R, Breuer T. Rotavirus serotype G9 is associated with more-severe disease in Latin America. Clin Infect Dis. 2006;43(3):312-4.

32. Clark HF, Lawley DA, Schaffer A, Patacsil JM, Marcello AE, Glass RI, et al. Assessment of the epidemic potential of a new strain of rotavirus associated with the novel G9 serotype which caused an outbreak in the United States for the first time in the 1995-1996 season. J Clin Microbiol. 2004;42(4):1434-8.

33. Albano F, Bruzzese E, Bella A, Cascio A, Titone L, Arista S, et al. Rotavirus and not age determines gastroenteritis severity in children: a hospital-based study. Eur J Pediatr. 2007;166(3):241-7.

34. Leshem E, Moritz RE, Curns AT, Zhou F, Tate JE, Lopman BA, et al. Rotavirus vaccines and health care utilization for diarrhea in the United States (2007-2011). Pediatrics. 2014;134(1):15-23.

35. de Rougemont A, Kaplon J, Lebon P, Huet F, Denis F, Alain S, et al. Unexpected substitution of dominant rotavirus G genotypes in French hospitalized children over five consecutive seasons. Eur J Clin Microbiol Infect Dis. 2009;28(4):403-7.

ACKNOWLEDGMENTS

We would like to thank Philip Bastable for editorial assistance. A part of the results of this study was presented on the 19th of May 2015 at the 6th European Rotavirus Biology Meeting in Dijon, France.

AUTHOR CONTRIBUTIONS STATEMENTS

AdR, JK and PP conceived and designed this study and revised the manuscripts. **JK and CF** performed the experiments. **MCLG, AMT, CP, AV, LMM, MC, RM, FH, FD, DH, ML, CB, AD, SA, JL, YG, BL, YM, FMS, AG, JG, VF, MR, VAF, SB, AGC, DG, PL, ML, PM, JFM, AS, GA, ABD, DO, RC, GL, OM, SP, BP, JL** collected the stool samples and the clinical data. **SA** performed the statistical analysis and modelling of the data. **AdR** analyzed the data and wrote the paper. All authors reviewed the manuscript.

ADDITIONAL INFORMATION

GenBank accession numbers: RVA/Human-wt/FRA/Lyon-R5957/2012/G12P[8]: KU291317, RVA/Human-wt/FRA/Poitiers-R5485/2012/G12P[8]: KU291318, RVA/Human-wt/FRA/Limoges-R6183/2012/G12P[8]: KU291319, RVA/Human-wt/FRA/Saint-Etienne-R6834/2013/G12P[8]: KU291320, RVA/Human-wt/FRA/Saint-Etienne-R6784/2013/G12P[8]: KU291321, RVA/Human-wt/FRA/Montpellier-R5396/2012/G12P[8]: KU291322, RVA/Human-wt/FRA/Orleans-R7653/2013/G12P[8]: KU291323, RVA/Human-wt/FRA/Montpellier-R7934/2014/G12P[8]: KU291324, RVA/Human-wt/FRA/Paris-R7590/2013/G12P[8]: KU291325, RVA/Human-wt/FRA/Caen-R6761/2013/G12P[8]: KU291326, RVA/Human-wt/FRA/Paris-R7128/2013/G12P[6]: KU291327, RVA/Human-wt/FRA/Paris-R6766/2013/G12P[8]: KU291328, RVA/Human-wt/FRA/Paris-R8857/2014/G12P[8]: KU291329, RVA/Human-wt/FRA/Orleans-R6110/2012/G12P[8]: KU291330, RVA/Human-wt/FRA/Paris-R6296/2012/G12P[8]: KU291331, RVA/Human-wt/FRA/Caen-R6679/2012/G12P[8]: KU291332, RVA/Human-wt/FRA/Paris-R7151/2013/G12P[8]: KU291333, RVA/Human-wt/FRA/Poitiers-R6940/2013/G12P[8]: KU291334, RVA/Human-wt/FRA/Brest-R6975/2013/G12P[8]: KU291335, RVA/Human-wt/FRA/Saint-Etienne-R8964/2014/G12P[8]: KU291336, RVA/Human-wt/FRA/Saint-Etienne-R1725/2007/G12P[6]: KU291337, RVA/Human-wt/FRA/Brest-R8391/2014/G12P[8]: KU291338, RVA/Human-wt/FRA/Brest-R8422/2014/G12P[8]: KU291339, RVA/Human-wt/FRA/Brest-R5875/2012/G12P[8]: KU291340, RVA/Human-wt/FRA/Lyon-R5790/2012/G12P[8]: KU291341, RVA/Human-wt/FRA/Lille-

R5374/2012/G12P[8]: KU291342, RVA/Human-wt/FRA/Lille-R6147/2012/G12P[8]: KU291343, RVA/Human-wt/FRA/Lille-R5373/2012/G12P[8]: KU291344, RVA/Human-wt/FRA/Saint-Etienne-R5462/2012/G12P[8]: KU291345, RVA/Human-wt/FRA/Caen-R6642/2012/G12P[8]: KU291346, RVA/Human-wt/FRA/Rennes-R5533/2012/G12P[8]: KU291347, RVA/Human-wt/FRA/Rennes-R5506/2012/G12P[8]: KU291348, RVA/Human-wt/FRA/Rennes-R5510/2012/G12P[8]: KU291349, RVA/Human-wt/FRA/Lyon-R5615/2012/G12P[8]: HF952916, RVA/Human-wt/FRA/Saint-Etienne-R4925/2011/G12P[8]: HF952915, RVA/Human-wt/FRA/Paris-R4380/2010/G12P[6]: HF952913 and RVA/Human-wt/FRA/Dijon-R4969/2011/G12P[8]: HF952914.

FIGURE LEGENDS

Figure 1. Temporal and geographical distribution of rotavirus infections in France from July 2009 to June 2014.

A. 5-year cumulative distribution of rotavirus infections per month in 7 major centres; **B.** Annual distribution of rotavirus infections per month of 7 major centres.

ONLINE APPENDIX FIGURE LEGENDS

Figure S1. Temporal distribution of group A rotavirus according to G genotype in France from 2009 to 2014.

A. 5-year cumulative distribution of G genotypes according to detection month ; B. 5-year cumulative prevalence of G genotypes according to detection month.

Figure S2. Distribution of RVA genotype combination over the last 13 epidemic seasons from 2001 to 2014.

Data from 2001 to 2009 were extracted from our previous studies for comparison [15, 35]. Detailed numbers of G12 strains for each season are indicated in the bottom table. * first detection of G12P[8]; ** emergence of G12P[8] strains in France.

Figure S3. Regional G12P[8] detection rate from 2009 to 2014.

Bars in red: G12 detection rates during emergence; bars in blue: G12 detection rates before and after emergence.

Figure S4. Maximum Likelihood phylogenetic tree based on the partial nucleotide sequences (575 nt) of the rotavirus G12 VP7 coding gene.

The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Reference strains retrieved from GenBank database are in bold-face. The French rotavirus G12 strains from this study are coloured according to the season of sample collection: brown for the 2009-2010 season, green for the 2010-2011 season, red for the 2011-2012 season, blue for the 2012-2013 season and pink for the 2013-2014 season. The following representative French rotavirus G12 strains from this study were submitted to the GenBank database.

509 **Figure S5. Fractional polynomial analysis of the relation between child age in month and**

510 **severity scores using a linear regression model including a non-zero intercept**.

511 IC95 is shown in grey

TABLE 1. Distribution and detection rates of G and P genotype combinations of group A rotavirus detected in France from 2009 to 2014

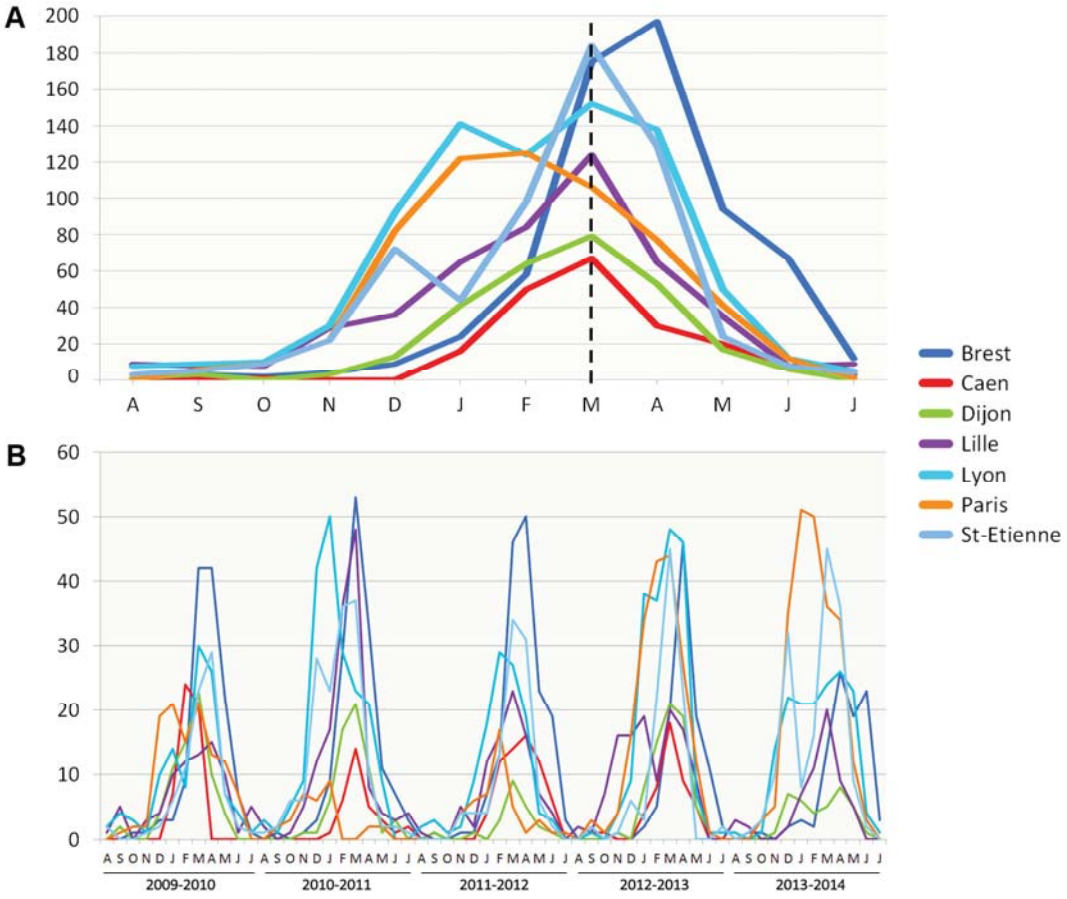
	No. of strains detected during the 5 consecutive seasons *(%)*										Total strains *(%)*	
	2009-2010 *n*=812		2010-2011 *n*=859		2011-2012 *n*=849		2012-2013 *n*=1095		2013-2014 *n*=1093		2009-2014 *n*=4708	
Common strains	**757**	***(93.2)***	**826**	***(96.2)***	**784**	***(92.3)***	**1011**	***(92.3)***	**1010**	***(92.4)***	**4388**	***(93.2)***
G1P[8]	503	*(61.9)*	628	*(73.1)*	465	*(54.8)*	731	*(66.8)*	593	*(54.3)*	2920	*(62.0)*
G2P[4]	143	*(17.5)*	37	*(4.3)*	50	*(5.9)*	43	*(3.9)*	12	*(1.1)*	285	*(6.1)*
G3P[8]	12	*(1.5)*	35	*(4.1)*	164	*(19.3)*	144	*(13.2)*	153	*(14.0)*	508	*(10.8)*
G4P[8]	16	*(2.0)*	63	*(7.3)*	7	*(0.8)*	24	*(2.2)*	12	*(1.1)*	122	*(2.6)*
G9P[8]	83	*(10.2)*	63	*(7.3)*	98	*(11.5)*	69	*(6.3)*	240	*(22.0)*	553	*(11.7)*
Emerging strains												
G12P[8]	**0**		**3**	***(0.3)***	**35**	***(4.1)***	**33**	***(3.0)***	**7**	***(0.6)***	**78**	***(1.7)***
Human reassortants	**28**	***(3.2)***	**11**	***(1.3)***	**17**	***(2.0)***	**11**	***(1.0)***	**7**	***(0.6)***	**74**	***(1.6)***
G1P[4]	5	*(0.6)*	4	*(0.5)*	4	*(0.5)*	0		0		13	*(0.3)*
G2P[8]	1	*(0.1)*	4	*(0.5)*	8	*(0.9)*	7	*(0.6)*	5	*(0.5)*	25	*(0.5)*
G3P[4]	21	*(2.4)*	1	*(0.1)*	1	*(0.1)*	0		1	*(0.1)*	24	*(0.5)*
G4P[4]	0		0		1	*(0.1)*	2	*(0.2)*	0		3	*(0.1)*
G9P[4]	1	*(0.1)*	2	*(0.2)*	3	*(0.4)*	2	*(0.2)*	1	*(0.1)*	9	*(0.2)*
Unusual strains	**11**	***(1.4)***	**5**	***(0.6)***	**4**	***(0.5)***	**12**	***(1.1)***	**8**	***(0.7)***	**40**	***(0.8)***
G1P[6]	1	*(0.1)*	0		0		5	*(0.5)*	0		6	*(0.1)*
G3P[3]	0		0		0		1	*(0.1)*	0		1	*(<0.1)*
G3P[6]	1	*(0.1)*	0		0		0		1	*(0.1)*	2	*(<0.1)*
G3/G9P[6] [a]	0		2	*(0.2)*	0		0		0		2	*(<0.1)*
G3P[9]	0		0		0		0		3	*(0.3)*	3	*(0.1)*
G4P[6]	2	*(0.2)*	0		0		0		0		2	*(<0.1)*
G6P[6]	1	*(0.1)*	1	*(0.1)*	2	*(0.2)*	2	*(0.2)*	4	*(0.4)*	10	*(0.2)*
G6P[14]	2	*(0.2)*	1	*(0.1)*	0		0		0		3	*(0.1)*
G8P[4]	1	*(0.1)*	0		0		0		0		1	*(<0.1)*
G8P[6]	1	*(0.1)*	0		0		0		0		1	*(<0.1)*
G8P[8]	0	*(0.1)*	1		0		1	*(0.1)*	0		2	*(<0.1)*
G8P[14]	1		0	*(0.1)*	1	*(0.1)*	0		0		2	*(<0.1)*
G10P[8]	0		0	*(0.1)*	1	*(0.1)*	0		0		1	*(<0.1)*
G12P[6]	1	*(0.1)*	0		0		3	*(0.3)*	0		4	*(0.1)*
Mixed infections [b]	**16**	***(2.0)***	**12**	***(1.4)***	**5**	***(0.6)***	**10**	***(0.9)***	**21**	***(1.9)***	**64**	***(1.4)***
Partially typed	**0**		**4**	***(0.5)***	**4**	***(0.5)***	**18**	***(1.6)***	**40**	***(3.7)***	**66**	***(1.4)***

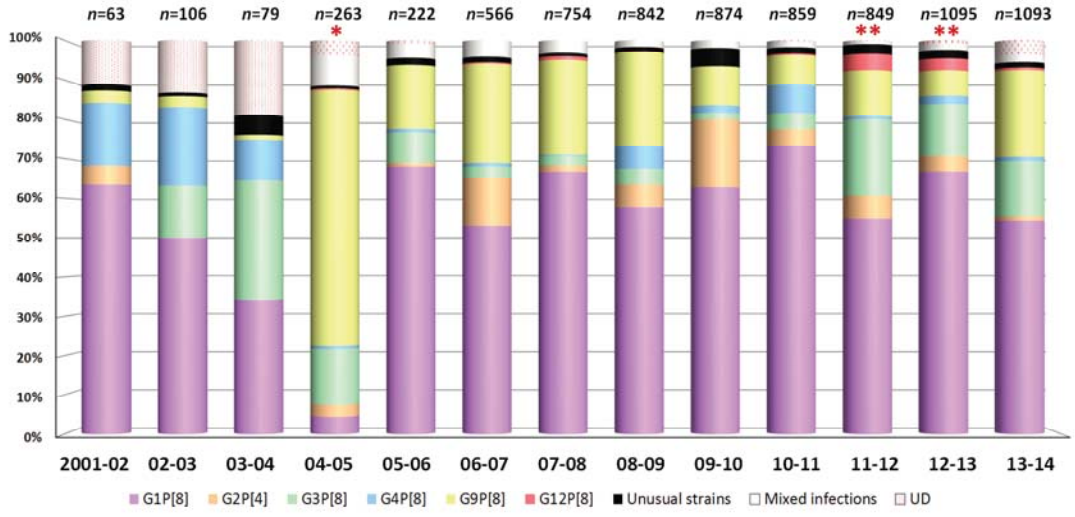
[a] These strains are also included in mixed infections; [b] Details on mixed infection strains are reported in Table S1.

TABLE 2. Clinical characteristics according to rotavirus genotypes in 624 hospitalized diarrheal children between 2009 and 2014

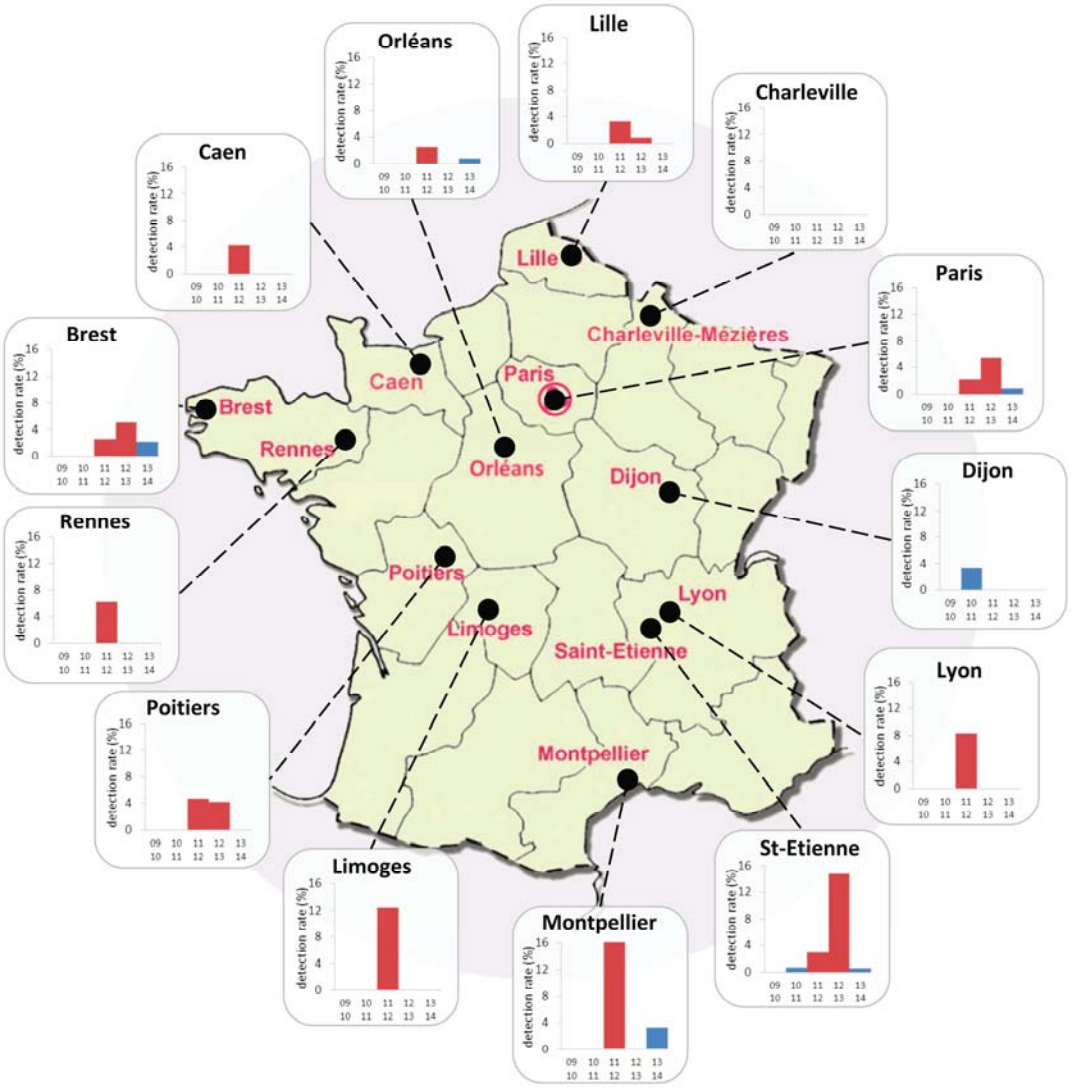
	G1P[8]	**G2P[4]**	**G3P[8]**	**G4P[8]**	**G9P[8]**	**G12P[8]**	**Total**	***P*** [a]	***P*** [b]
	***n*=407**	***n*=59**	***n*=74**	***n*=5**	***n*=52**	***n*=27**	***n*=624**		
Prevalence	64.2%	9.3%	11.7%	0.8%	8.2%	4.3%	**100%**	-	-
Mean age, months	14.2 ±11.0	15.6 ±13.4	15.6 ±11.9	17.5 ±15.6	11.9 ±8.8	15.5 ±11.2	**14.3 ±11.0**	0.512 [d]	0.675 [d]
Sex ratio	1.6	1.3	1.3	0.7	2.5	1.8	**1.6**	0.520 [e]	0.495 [e]
Diarrhea	93.9%	94.9%	97.3%	100.0%	96.2%	96.0%	**94.7%**	0.607 [e]	0.962 [e]
Mean number of diarrhea bouts per day	6.0 ±3.9	4.5 ±3.0	8.3 ±5.9	6.4 ±5.9	7.5 ±3.3	5.9 ±5.8	**6.4 ±4.3**	0.433 [d]	0.078 [d]
Vomiting	80.0%	84.7%	89.0%	100.0%	78.8%	80.0%	**81.4%**	0.575 [e]	0.498 [e]
Mean number of vomiting bouts per day	4.6 ±3.4	5.3 ±5.5	6.1 ±4.0	2.5 ±0.7	5.8 ±5.2	7.7 ±6.3	**5.1 ±4.2**	0.114 [d]	0.516 [d]
Blood in stool	1.2%	0.0%	0.0%	0.0%	0.0%	4.0%	**1.0%**	0.306 [e]	0.464 [e]
Fever >38.5°C	72.3%	73.9%	61.0%	75.0%	64.3%	77.8%	**70.5%**	0.349 [e]	0.361 [e]
Temperature, °C	38.5 ±1.0	38.6 ±0.9	38.3 ±1.0	38.4 ±0.1	38.3 ±0.8	38.5 ±0.9	**38.5 ±0.9**	0.719 [d]	0.308 [d]
Dehydration	54.7%	53.4%	75.7%	40.0%	65.4%	56.0%	**57.7%**	0.139 [e]	0.131 [e]
Dehydration degree, %	7.2 ±3.5	6.0 ±2.5	7.0 ±3.4	3.5 ±2.1	6.6 ±3.6	6.4 ±2.8	**6.9 ±3.4**	0.545 [d]	0.223 [d]
Hypovolemia	37.9%	25.9%	64.8%	20.0%	46.0%	54.2%	**41.0%**	0.345 [e]	0.158 [e]
Intravenous rehydration	61.2%	65.5%	74.3%	40.0%	60.8%	68.0%	**63.2%**	0.337 [e]	0.265 [e]
Oral rehydration	13.6%	19.0%	12.2%	20.0%	21.6%	12.0%	**14.4%**	0.553 [e]	0.402 [e]
Intensive care	1.2%	1.7%	2.7%	0.0%	1.9%	0.0%	**1.4%**	0.740 [e]	0.628 [e]
Hospitalization duration, days	2.8 ±1.7	2.8 ±1.6	2.7 ±1.4	1.8 ±1.1	2.5 ±1.5	3.2 ±1.7	**2.8 ±1.6**	0.372 [d]	0.550 [d]

[a] Comparison between G1P[8] and G12P[8] groups; [b] Comparison between all genotype groups; [c] Comparison within all genotype groups; [d] using Kruskal-Wallis test; [e] using Fisher's exact-test; Statistically significant if P<0.05.





G12P[8]	-	-	-	1	1	2	7	1	0	3	35	33	7
G12P[6]	-	-	-	-	-	-	1	-	1	-	-	3	-


Orléans
Lille
Charleville
Caen
Paris
Brest
Dijon
Rennes
Lyon
Poitiers
St-Etienne
Limoges
Montpellier
Lille
Charleville-Mézières
Caen
Paris
Brest
Rennes
Orléans
Dijon
Poitiers
Lyon
Limoges
Saint-Etienne
Montpellier
detection rate (%)
09 10 11 12 13 14