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Predominance of rotavirus G9 genotype in children hospitalized for rotavirus gastroenteritis in Belgium during 1999–2003

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Abstract

Background: Group A rotavirus genotypes G1, G2, G3 and G4 are the main etiological agents of infantile diarrhea. The G9 rotavirus has recently emerged as a fifth important genotype all over the world.
Objective: To characterize the VP7 gene of group A rotaviruses from gastroenteritis patients admitted to the Gasthuisberg University Hospital, Leuven, Belgium, during 1999–2003.
Study design: Rotavirus antigen was detected in stool specimens using an enzyme immunoassay. G-typing was performed by reverse transcriptase polymerase chain reaction (RT-PCR) amplification and sequencing of the complete VP7 gene.
Results: The genotype distribution varied markedly over the four rotavirus years in Belgium. In the 1999–2000 rotavirus year, G1 was the predominating type (72%), and G9 was present in 5% of the rotavirus-positive patients. In the 2000–2001 and 2002–2003 years, G9 appeared as the dominating strain (45% and 53%, respectively). In the 2001–2002 year, between two G9 epidemic years, G1 was dominating (66%) but G9 was still present in 24%. All the G9 isolates were combined with P[8] and shared a high gene sequence similarity (<3% sequence divergence on the nucleotide and amino acid level). Phylogenetic analysis of the VP7 genes revealed that our Belgian G9 strains clustered together with recent G9 strains from all over the world, distinct from the prototype G9 strains isolated in the 1980s.
Conclusion: Our study indicates that although the first introduction of G9 isolates in the Belgian population was recorded in 1997, G9 strains were able to establish themselves quickly as the predominant genotype. The emergence of G9 as an important pathogen in both developing as industrialized countries necessitates the urgent consideration of the G9 moiety in rotavirus vaccines.

Keywords: Rotavirus; Genotype; G9; Phylogeny

1. Introduction

Rotaviruses are the major etiological agents of severe infantile diarrhea worldwide, and cause approximately 2 million hospitalizations and 352.000–592.000 deaths in children <5 years of age each year (Parashar et al., 2003). Rotaviruses belong to the Reoviridae family and are composed of three protein layers surrounding the 11 segments of double-stranded RNA. The rotaviral outer capsid is composed of the VP7 and VP4 proteins. A dual classification system has been established for rotaviruses, with the VP7 glycoprotein defining G-types and the protease sensitive VP4 defining P-types. At least 15 G-types and 23 P-types have thus far been characterized, and various combinations of G- and P-types exist. Since most rotavirus strains isolated during the 1990s belonged to G1P[8], G2P[4], G3P[8] and G4P[8], current candidate rotavirus vaccines only target the G1 to G4 genotypes.

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The capacity of these first-generation vaccines to provide heterotypic protection against other G-types is unclear.

G9 rotavirus strains have been associated with infections in humans, but seldom in animals (Hoshino and Kapikian, 1996). Until 1994, G9 strains had been isolated only sporadically (Nakagomi et al., 1990; Ramachandran et al., 1996) since this first discovery (WI61, G9P[8]) in 1983 in Philadelphia, USA (Clark et al., 1987). The emergence of G9 strains has subsequently been documented in many countries across all continents, and they are now recognized as the fifth globally important rotavirus genotype. The presence of G9 rotavirus has also been reported in European countries such as Belgium, France, Holland, Ireland, Italy, Scotland and UK (Bon et al., 2000; Cubitt et al., 2000; Fitzgerald et al., 1995; Iturriza-Gomara et al., 2000; Martella et al., 2003; O'Halloran et al., 2000; Van der Donck et al., 2003; Widdowson et al., 2000). It is also interesting to note that G9 rotaviruses were isolated in a significant percentage of sewage samples (6%) from Barcelona, Spain (Villena et al., 2003).

The G9 strains are of particular importance in terms of virus evolution because they exhibit a high level of reassortment (Jain et al., 2001; Unicomb et al., 1999). They showed a variety of combinations with the VP4 gene segments P[8], P[6], P[11], P[4], and P[19], although the predominant strain is G9P[8] (Adah et al., 2001; Ramachandran et al., 2000; Santos et al., 2001; Zhou et al., 2001). Analysis of a large collection of VP7 sequences from different countries over an extended time period suggested that the differences shown in the nucleotide sequences at the antigenic sites divide serotypes into different monotypes or lineages (Hoshino et al., 1994; Jin et al., 1996; Kirkwood et al., 1993).

The rotavirus year in Belgium starts from August and ends in July. In this study, a total of 628 rotavirus samples from gastroenteritis patients admitted between 1999 and 2003 to the Gasthuisberg University Hospital, Leuven, Belgium, were G-typed using molecular methods. The aim of the study was to examine the VP7 genes of the local G9 strains and to find out their evolutionary relationship with other G9 strains circulating all over the world.

2. Materials and methods

2.1. Rotavirus antigen detection

Rotavirus antigens were detected using a solid phase sandwich type enzyme immunoassay (DAKO Ltd., Cambridgeshire, UK) in stool specimens. An aliquot of a faecal suspension was added to a plastic microtiter well coated with a monoclonal antibody directed against the rotavirus group-specific antigen VP6 protein. The solution was simultaneously incubated with an anti-rotavirus monoclonal antibody conjugated to horseradish peroxidase, resulting in the rotavirus antigen being sandwiched between the solid-phase and the enzyme-linked antibodies. After 60 min incubation at room temperature, the sample well was washed to remove unbound enzyme-labeled antibodies. Urea peroxide and tetramethylbenzidine were added as substrate and incubated for 10 min at room temperature. The enzymatic reaction that converts the colorless substrate to a blue color was stopped with 1N H_2SO_4, and the absorbance was determined spectrophotometrically. Specimens with absorbance units (A_{450}) greater than 0.150 were considered positive.

2.2. RNA extraction

Viral RNA was extracted using the QIAamp Viral RNA mini kit (Qiagen/Westburg, Leusden, The Netherlands) according to the manufacturer's instructions.

2.3. RT-PCR

The extracted RNA was denatured at 97 °C for 5 min. G and P genotyping were performed as previously described by Gentsch et al. (1992) and Gouvea et al. (1990), respectively. Reverse transcription-PCR (RT-PCR) was carried out using the Qiagen OneStep RT-PCR Kit (Qiagen/Westburg). For G-typing, we amplified a 1062 bp fragment of the VP7 gene with the forward primer Beg9 (5′-GGCTTTAAAAGAGAGAATTTCCGTCTGG-3′; prototype strain Wa, GenBank accession number M21843, nucleotides (nt) 1–28) and the reverse primer End9 (5′-GGTCACATCATACAATTCTAATCTAAG-3′; prototype strain SA11, accession number K02028, nt 1062–1036). For P genotyping, the primers Con3 (5′-TGGCTTCGCCATTTTATAGACA-3′; prototype strain KU, accession number M21014, nt 11–32) and Con2 (5′-ATTTCGGACCATTTATAACC-3′; prototype strain KU, nt 887–868) were used to amplify an 876 bp fragment of the entire VP8* fragment and the first 40 amino acids of the VP5* fragment of the rotavirus VP4 gene. The reaction was carried out with an initial reverse transcription step at 45 °C for 30 min, followed by PCR activation at 95 °C for 15 min, 35 cycles of amplification (30 s at 94 °C, 45 s at 53 °C, 1 min at 72 °C), and a final extension of 7 min at 72 °C in a GeneAmp PCR System 9700 thermal cycler (Perkin-Elmer, Foster City, CA, USA). PCR products were run on a polyacrylamide gel, stained with ethidium bromide and visualized under UV-light.

2.4. Nucleotide sequencing

The PCR amplicons were purified with the QIAquick PCR purification kit (Qiagen/Westburg), and sequenced in both directions using the dideoxynucleotide chain termination method with the ABI PRISM® BigDye Terminator Cycle Sequencing Reaction kit (Perkin-Elmer Applied Biosystems, Foster City, CA) on an automated sequencer (ABI PRISM™ 3100) at the Rega Institute core sequencing facility. The same primers described above for the amplification of VP7 and VP4 genes were also used as sequencing primers.

2.5. Polyacrylamide gel electrophoresis (PAGE)

G-untypeable samples were investigated using polyacrylamide gel electrophoresis (PAGE) and silver staining as described by Herring et al. (1982).

2.6. DNA and protein sequence analysis

The chromatogram sequencing files were inspected using Chromas 2.23 (Technelysium, Qld, Australia), and contigs were prepared using SeqMan II (DNASTAR, Madison, WI). Nucleotide and protein sequence similarity searches were performed using the National Center for Biotechnology Information (NCBI, National Institutes of Health, Bethesda, MD) BLAST (Basic Local Alignment Search Tool) server on GenBank database, release 138.0 (Altschul et al., 1990). Multiple sequence alignments were calculated using CLUSTALX 1.81 (Thompson et al., 1997). Sequences were manually edited in the GeneDoc version 2.6.002 alignment editor.

2.7. Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 2.1 software package (Kumar et al., 2001). Genetic distances were calculated using the Kimura-2 parameter. The dendrogram was constructed using the neighbor-joining method.

2.8. Data analysis

Data were analyzed by SPSS for Windows, release 11.5.1 (LEAD Technologies Inc., Charlotte, NC, USA).

2.9. DNA sequence submission

The nucleotide sequence data reported in this paper were deposited in GenBank using the National Center for Biotechnology Information (NCBI, Bethesda, MD) Sequin, version 5.00 under accession numbers AY487853–AY487895 for the VP7 sequences.

3. Results

Samples were collected during four complete rotavirus years, 1 August 1999 to 31 July 2000 (168 patients), 1 August 2000 to 31 July 2001 (112 patients), 1 August 2001 to 31 July 2002 (115 patients) and 1 August 2002 to 31 July 2003 (233 patients). The age range of the patients was 0.2–81.5 months, median age 11 months and mean age 12.7 months. There was no significant difference concerning the age observed between the patients infected by G9 or the other types ($P > 0.05$) (Fig. 1). Seventy-five percent of the rotavirus incidence was recorded between January and April with seasonal peaks in February or March. In Belgium these months coincide with the end of winter and the beginning of spring, with average temperatures between 3.3 and 6.6 °C (Fig. 2). Rotavirus infections are rarely detected (2.4%) in July to September with average temperatures of 16–18 °C.

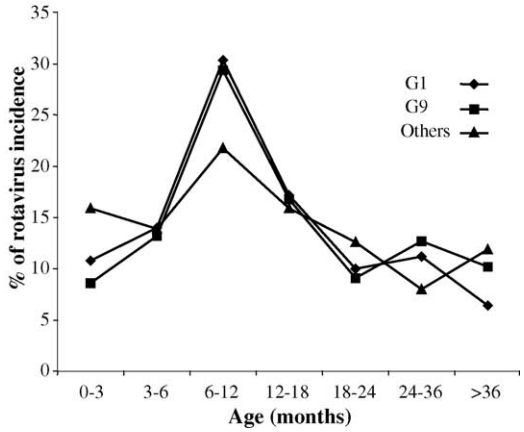

Fig. 1. Summary of rotavirus G-types detected in different age groups.

3.1. Distribution of G and P types

A total of 628 out of 641 (98%) rotavirus antigen-positive samples could be G-typed by RT-PCR combined with sequencing of the VP7 gene segment. The untypeable samples were investigated using PAGE and silver staining, but were found negative for rotaviral RNA, indicating that these samples were false positive with the enzyme immunoassay. Therefore these samples were excluded for further evaluation. Considerable genotype fluctuations were found over the 4 years (Fig. 3). Overall, G1 was the most prevalent genotype during the four rotavirus years (41.6%). G9 strains were present in 32.6% of the samples, G3 in 9.9%, G2 in 9.7%, G4 in 5.9%, G6 in 0.2%, and G8 in 0.3%. In the 1999–2000 rotavirus year, G1 was the predominating type (72%) followed by G2 (19%), and G9 was present in only 5% of the rotavirus-positive patients. Interestingly, in the 2000–2001 and 2002–2003 rotavirus years, G9 was the most prevalent genotype representing 45% and 52% of the patients respec-

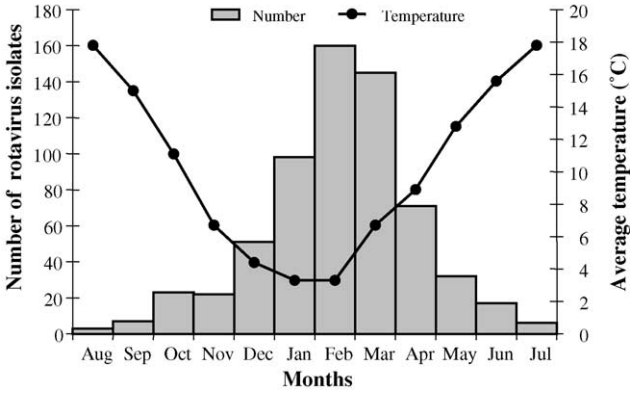

Fig. 2. Seasonal pattern of rotavirus detection. Average temperature data were obtained from the Royal Meteorological Institute of Belgium, Brussels.

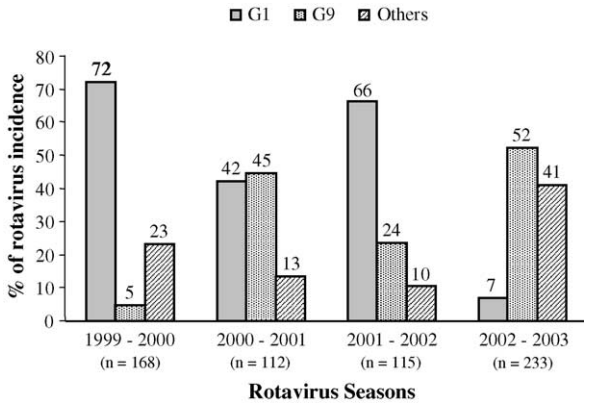

Fig. 3. Fluctuation of rotavirus G-genotypes during 4 years (1999–2003).

Table 1
Distribution of rotavirus G types in Belgium during the 1999–2003 rotavirus years

G type	Percentage of rotavirus incidence			
	1999–2000	2000–2001	2001–2002	2002–2003
G1	72.0	42.0	66.1	7.3
G2	18.4	12.5	2.6	5.6
G3	1.8	0.0	1.7	24.4
G4	3.0	0.9	4.3	11.2
G6	0.0	0.0	0.9	0.0
G8	0.0	0.0	0.9	0.0
G9	4.8	44.6	23.5	51.5

tively. In 2001–2002, between the two G9 epidemic years, G1 was dominating (66%), but G9 was still present in 24% of the cases. A significantly higher incidence of rotavirus infections with G3 and G4 strains was observed in the 2002–2003 year compared to the previous years (Table 1).The prevalence of G1 and G2 decreased markedly in the last rotavirus year. It can be suggested that dominant strains can be abruptly replaced by other less common strains over time. G1, G3, G4

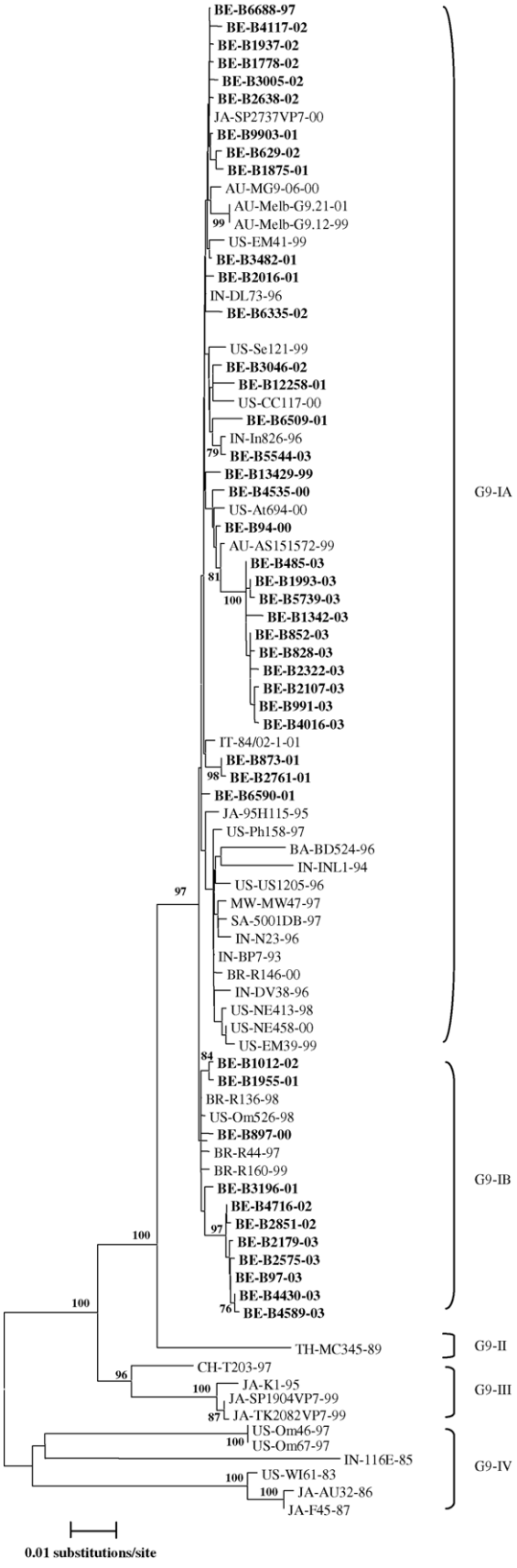

Fig. 4. Neighbor-joining phylogenetic tree based on nucleotide sequences of the VP7 encoding genes for Belgian G9 strains and other established rotavirus G9 strains from all over the world. The VP7 sequences were obtained from published reports and the GenBank database. AU, Australia; BA, Bangladesh; BE, Belgium; BR, Brazil; CH, China; IN, India; JA, Japan; MW, Malawi; SA, Republic of South Africa; TH, Thailand; US, United States. The GenBank accession numbers were: SP2737VP7 (AB091752), MG9-06 (AY307085), Melb-G9.21 (AY307090), Melb-G9.12 (AY307088), EM41 (AJ491170), DL73 (AJ491165), Se121 (AJ491192), CC117 (AJ491153), In826 (AJ491173), At694 (AJ491159), 95H115 (AB045373), Ph158 (AJ491183), BD524 (AJ250543), INL1 (AJ250277), US1205 (AF060487), MW47 (AJ250544), 50001DB (AF529864), N23 (AJ491177), BP7 (AJ491161), R146 (AF274970), DV38 (AJ491168), NE413 (AJ491178), NE458 (AJ491180), EM39 (AJ491169), R136 (AF438228), Om526 (AJ491182), R44 (AF438227), R160 (AF274971), MC345 (D38055), T203 (AY003871), K1 (AB045374), SP1904VP7 (AB091754), TK2082VP7 (AB091755), Om46 (AJ491181), Om67 (AJ491179), AU32 (AB045372), 116E (L14072). The VP7 sequences of WI61 and F45 were obtained from Green et al. (1989). The number adjacent to the nodes represents the percentage bootstrap support (of 1000 replicates) for the clusters to the right of the node. Bootstrap values lower than 75% were not shown.

and G9 strains always contained P[8] specificity, except one G3 with P[14]. G2 strains were combined with P[4] in all cases.

3.2. Molecular characterization of G9 strains

Our main goal was to characterize G9 strains isolated in Belgium by sequencing their VP7 genes and to compare them with other G9 strains circulating in different countries. We aligned the nucleotide sequences of the VP7 gene of all our G9 strains ($n = 205$). Most of the VP7 genes of our strains had nucleotide and deduced amino acid sequences that were very closely related to each other or virtually identical. We found less than <3% nucleotide diversity among these strains. The VP7 nucleotide sequences of the G9 strains were most similar (99–100% nt identities) with Australian strain AS151572 (G9P[8], isolated in 1999), American Strain AT694 (G9P[8], 2000–2001), Japanese strain 00-SP2737VP7 (G9P[8], 2000), Brazilian Strain R136 (G9P[8], 1998) and Indian strain DL73 (G9P[8], 1996–1998). They showed only 92% and 94% amino acid homology in their VP7 gene sequence with the Indian neonatal G9 reference strain 116E and the prototype US strain WI61, respectively. All Belgian G9 strains contained the P[8] specificity of the VP4 protein that was most similar (99% nt identity) with the Malawi strain OP351 (G1P[8], 1998).

3.3. Phylogenetic analysis of G9 strains

A dendrogram was constructed with the nucleotide sequences of all different G9 strains isolated in Belgium and other recent and prototype G9 strains from other parts of the world (Fig. 4). The phylogenetic tree identified four major lineages: G9-I, G9-II, G9-III and G9-IV (Fig. 4). Phylogenetic analysis clearly demonstrates that the most recent emerging strains including the Belgian G9 strains clustered together in the G9-I lineage regardless of where and when they were isolated. Lineage I can also be divided into two sublineages (G9-IA and G9-IB). Belgian strains clustered in both G9-IA and G9-IB lineages with G9 strains from different countries all over the world, i.e. Japan, Italy, Bangladesh, Australia, India, Africa, US and Brazil. They are more closely related to each other than to G9 reference strains isolated in the 1980s (WI61, 116E, AU32 and F45 in lineage IV). Some of the G9 strains isolated in China (T203, 1997; lineage III), Japan (K1, 1995; SP1904VP7, 1999; lineage III) and Thailand (MC345, lineage II) were placed between the recent and the prototype clusters.

4. Discussion

Our study on the molecular epidemiology of rotaviruses is the most systematic study of rotavirus diversity in Belgium encompassing several consecutive rotavirus seasons. We provide the description in an industrialized country of rotavirus seasons in which G9 was the most prevalent genotype. Only one G9 rotavirus strain was isolated in 1997 in Belgium and it has become the most prevalent type in the following years with the highest incidence (51%) in the 2002–2003 rotavirus year. The substantial numbers of G9 strains (205 isolates) allowed us a thorough investigation of their genetic characteristics.

First, it can be speculated that the G9P[6] strain is the ancestor of the current Belgian G9P[8] strain. After going through reassortment events with the most common circulating strains in Europe (G1P[8], G3P[8] and G4P[8]), they acquired the P[8] VP4 specificity and became the predominant strain in the community. G9P[6] was first isolated in Europe (UK) in the 1995–1996 year and was replaced by G9P[8] in the following years (Iturriza-Gomara et al., 2000). The replacement of P[6] with P[8] can be explained by the replicating disadvantage of the VP4 gene with P[6] specificity in humans, because P[6] is thought to be of animal origin. G9P[6] rotaviruses were also found in a nursery outbreak in The Netherlands (Widdowson et al., 2000) in 1999, which indicated the lack of maternal antibodies against P[6] in the neonates. This finding indicates that G9 rotaviruses have been introduced only recently in the European population.

Secondly, all Belgian G9 strains contained the P[8] specificity, the most common P-type isolated in the industrialized countries, i.e. Italy, Ireland, France and Australia (Bon et al., 2000; Martella et al., 2003; O'Halloran et al., 2000; Palombo et al., 2000). However, in developing countries like India, Bangladesh and African countries, both G9P[6] and G9P[8] strains have been co-circulating, which can also be explained by the frequent reassortment between different strains and by a high percentage of mixed infections in these countries (Jain et al., 2001; Laird et al., 2003; Ramachandran et al., 1998; Steele and Ivanoff, 2003; Unicomb et al., 1999). It is possible that the G9 strains in industrialized countries are genetically more stable because of less frequent reassortment events.

Finally, molecular characterization of G9 strains circulating all over the world indicates that rotavirus G9 strains isolated thus far can be divided into two major groups: prototype strains and recent strains. The unique nature of the recent G9 strains which are only distantly related to prototype strains at the VP7 gene sequence level, raises questions concerning the origin and evolution of the recent strains: did they evolve from prototype strains or do they represent an independent recent introduction of a novel G9 strain? By comparing the VP7 gene sequences of these two lineages, several distinct amino acid substitutions in the major antigenic epitope sites were observed (amino acid position 87, 208, 220 and 242).

In summary, the phylogenetic analysis of the worldwide distribution of the G9 strains illustrates the almost total absence of locally restricted rotavirus lineages. This globalization of the rotavirus strains might be the results of extensive transport, travel, and migration in the global village. The suboptimal performance of rotavirus vaccines in trials carried out in developing countries indicates that the precise identification of the rotavirus genotype spectrum will be necessary

for improvements in rotavirus vaccine design (De Mol et al., 1986; Hanlon et al., 1987; Linhares et al., 1999). None of the current candidate vaccines in clinical trials incorporate the G9 moiety. The recent emergence of G9 as an important pathogen in both developing and industrialized countries necessitates the inclusion of G9 in future rotavirus vaccines. It is likely that second generation rotavirus vaccines will need to be regularly tailored to reflect the temporal and spatial genotype fluctuations, much in the same way as influenza vaccines are specifically formulated for both hemispheres before each flu season.

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