Emergence of human rotavirus genotype G9 in metropolitan Detroit between 2007 and 2009

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Between January 2007 and April 2009, rotavirus (RV)-positive stool samples from 238 children with acute gastroenteritis, seen at Children's Hospital of Michigan in Detroit, USA, were collected and RV genotyping was performed. G and P genotypes were determined by RT-PCR and nucleotide sequencing was conducted on selected G9 and P[6] strains. Correlation between the severity of gastroenteritis episode and the infecting G genotype was done using a 14-point scoring system. The predominant G genotype was G9 (39.5 %), followed by G1 (35.3 %) and G4 (15.5 %), while P[8] was the most prevalent P genotype (66.5 %), followed by P[4] (21.9 %) and P[6] (11.2 %). The gene combinations G1P[8] and G9P[8] were the most prevalent (21.4 % and 20.6 %, respectively), followed by G4P[8] (13 %) and G9P[6] (8.8 %). Immunization data showed that only 17/238 (7.1 %) children received $\geq$ one dose of RV vaccine (the pentavalent vaccine RotaTeq or the monovalent vaccine Rotarix) and that 10/17 were infected with G4P[8] strains. Severity of RV gastroenteritis episodes was not related to the infecting G genotype. Our results suggest a high proportion of genotype G9 strains in combination with P[8], P[6] and P[4] specificity circulating in the metropolitan Detroit area. While the protective efficacy of the RV vaccines has been demonstrated against G9P[8] strains, the level of cross-protection offered by the vaccines against G9 strains with P[6] and P[4] genotypes in the Detroit paediatric population remains to be determined.

Received 11 October 2010

Accepted 1 March 2011

INTRODUCTION

Rotavirus (RV) is a major cause of acute gastroenteritis worldwide. It accounts for approximately 40 % of diarrhoea hospitalizations in children <5 years of age (Parashar *et al.*, 2006). An estimated 527 000 RV-infected children younger than 5 years die each year, the majority in developing countries (Parashar *et al.*, 2003). Control of RV disease is most likely to be achieved through widespread use of an effective vaccine.

The outer layer of group A RV is composed of two proteins, VP4 and the VP7, which are encoded by RNA segment 9 and 4, respectively (Estes & Kapikian, 2007). Rotaviruses are classified into G and P types based on the reactivity of the

outer capsid proteins, VP7 and VP4, with serotype-specific monoclonal antibodies or on the amplification of PCR amplicons using genotype-specific primers. Genotype P[8] is the most common P type globally, followed by P[4] and P[6]. P[8] tends to be more frequently associated with serotypes G1, G3 and G4, while P[4] is more likely to be associated with serotype G2. Prior to 1990, the most common serotypes globally were G1P[8], G2P[4], G3P[8] and G4P[8]; since then, G9P[8] has been recognized as the fifth most globally common type (Gentsch *et al.*, 1992, 2009; Koshimura *et al.*, 2000). The G and P type specific antibodies, particularly those to the G antigen, are believed to play an important role in protection against RV gastroenteritis (Gentsch *et al.*, 2005). This prompted the development of vaccines based on the most common circulating strains in target populations at the time: G1, G2, G3 and G4. Although the first licensed RV vaccines were designed to cover the most common G types, the current RV vaccines were designed to cover the most common G and P types (Gentsch *et al.*, 2005).

Abbreviation: CDC, Centers for Disease Control and Prevention.

The GenBank/EMBL/DDBJ accession numbers for the VP7 gene sequence of G9 strains isolated in this study are HQ018932, HQ018933 and HQ018934.

The RV vaccine strategy is based on eliciting immunity in recipients comparable to that induced by natural infection through production of homotypic and heterotypic antibodies to serotypes targeted by the vaccine. Presently, two oral RV vaccines are approved by the Federal Drug Administration of the USA for administration to infants. A pentavalent human-bovine reassortant vaccine, Rotateq, was developed by Merck and contains the four major G serotypes G1, G2, G3 and G4 with the most predominant P type, P[8] (RV5). A monovalent attenuated human vaccine, Rotarix, was developed by GlaxoSmithKline Biologicals and contains a G1P[8] strain (RV1).

Studies from different parts of the world suggest that serotypes other than G1–G4 are important causes of RV gastroenteritis. These include G5 in Brazil and G8 in Malawi and other parts of Africa (Cunliffe *et al.*, 2001; Leite *et al.*, 1996). G9 strains emerged in 1993 as one of five globally prevalent genotypes and in 2001 accounted for a rate of at least 5.8% of RV cases (Laird *et al.*, 2003). However, this may not reflect the current prevalence of the G9 genotype, since recent regional epidemiological studies have shown prevalence of G9 genotypes as high as 50–90% in some settings (de Rougemont *et al.*, 2009; Rodrigues *et al.*, 2007; Sánchez-Fauquier *et al.*, 2006).

In 2001, we genotyped RV strains recovered from 100 children with acute gastroenteritis seen at the Children's Hospital of Michigan in Detroit, MI, USA. G1 was the predominant genotype (73%), followed by G4 (17.8%). Genotype G9, not present in the current vaccine formulations, was detected at very low frequencies in the USA in the late 1990s and in the Detroit study in 7% of patients (Abdel-Haq *et al.*, 2003). Whether widespread implementation of RV vaccines will cause emergence of non-vaccine serotypes was unclear.

Genotyping of circulating RV strains has important implications on the protective efficacy of the current vaccine formulations and monitoring of vaccine impact. Although natural RV infection in infants confers protection against subsequent infection (Velázquez *et al.*, 1996), RV vaccines are most effective when they include viral serotypes similar to those circulating in the community (homotypic responses). Vaccines with non-matching circulating serotypes induce heterotypic responses that are believed to be less protective (Perez-Schael *et al.*, 1990). This study was undertaken at the Children's Hospital of Michigan to monitor the prevalence of RV genotypes in metropolitan Detroit and to detect possible replacement of the predominant serotypes following RV vaccine licensing in February 2006. We also studied the correlation between the specific causative RV genotype and severity of gastroenteritis episodes.

METHODS

Patients. Between January 2007 and April 2009, stool specimens were collected from infants and children under 6 years of age

presenting with acute gastroenteritis to the Children's Hospital of Michigan. The hospital has a 230-bed capacity and is a tertiary care teaching institution. Stools were tested for RV antigen using the enzyme immunoassay detection kit Sure-Vue Rota Test (Fisher HealthCare), and stored at -80°C in 10% balanced salt solution until RNA extraction. The study was approved by the Human Investigation Committee at Wayne State University.

RNA extraction, and G and P typing by RT-PCR. RV RNA from stool samples was extracted using TRIzol Reagent according to the manufacturer's recommendation (Sigma-Aldrich). RNA was stored at -80°C in nuclease-free water until further use. Reverse transcription was performed by using Ready-to-Go RT-PCR beads (GE Healthcare) using random hexamer primers.

RV G and P types were determined using two-step, primer-specific, semi-nested multiplex PCR. For G typing, PCR amplification was performed using VP7 forward and VP7 reverse primers. The second step multiplex PCR was performed using VP7 reverse and G1, G2, G3, G4, G9 and G10 specific primers (Gouvea *et al.*, 1990; Iturriza-Gómara *et al.*, 2004). P typing was also performed as a two-step PCR employing Con2 and Con3 for the first PCR for amplification of the VP4 segment, and Con3, P[4], P[6], P[8], P[9], P[10] and P[11] specific primers for the second multiplex PCR (Gentsch *et al.*, 1992; Iturriza-Gómara *et al.*, 2004). PCR amplifications were performed by using PCR reagents (Bio-Rad) and amplification conditions described previously (Iturriza-Gómara *et al.*, 2004). The resulting amplicons were analysed on a 2% agarose gel stained with ethidium bromide. G and P genotypes were determined on the basis of amplicon sizes (Gentsch *et al.*, 1992; Gouvea *et al.*, 1990; Iturriza-Gómara *et al.*, 2004).

Sequence alignments and phylogenetic analysis. Representative G9 and P[6] strains were sequenced by using the primers Beg9 and End9 to sequence G types (Gouvea *et al.*, 1990), and the primers Con2 and Con3 to sequence P types (Gentsch *et al.*, 1992). The PCR products were purified using a Spin Column Gel Extraction kit (Qiagen) and used as template for sequencing by Big-Dye Terminator cycle sequence reaction in an automated ABI DNA sequence analyser (Applied Biosystems). Multiple alignments and phylogenetic analysis were performed using UPGMA and neighbour-joining methods using CLC sequence analysis software (CLC bio).

Clinical data. Clinical data were collected retrospectively. A 14-point scoring system modified by Cascio and colleagues was used to classify the severity of RV disease as shown in Table 1 (Ansaldi *et al.*, 2007; Cascio *et al.*, 2001). RV vaccination status was recorded by accessing the Michigan Care Improvement Registry.

Statistical analysis. Statistical analyses of clinical variables and genotype frequencies were performed using SPSS version 17.0. The non-parametric Kruskal–Wallis one-way analysis of variance by ranks procedure was employed for testing equality of population medians of severity scores among genotype groups, followed by pairwise examinations using the non-parametric Mann–Whitney test for unpaired data. In all comparisons, statistically significant differences were considered achieved at a P -value ≤ 0.05 , two-tailed.

RESULTS

For the 36-month study period between 2007 and 2009, RV was detected by enzyme immunoassay in 304 stool samples in 2007, in 95 stool samples in 2008 and in 230 stool samples in 2009. A total of 238 (38%) RV-positive stool samples were randomly selected from 2007–2009 and genotyped. The age range of these children was 11 days–70

Table 1. Classification system used to score RV gastroenteritis disease severity

Symptom	Occurrence	Score
Diarrhoea – duration	<2 days	1
	2–4 days	2
	>4 days	3
Diarrhoea – number of stools	Three stools	1
	Four to five stools	2
	>Five stools	3
Vomiting and duration	None	0
	1–2 days	1
	>2 days	3
Fever (temperature >38.2 °C)	No	0
	Yes	2
Intravenous hydration required	No	0
	Yes	3

months (mean 18 months, median 16 months). Of these, 57 % were boys and 36 % were younger than 1 year.

Over the study period, G9 was the predominant G genotype (39.5 %), followed by G1 (35.3 %), G4 (15.5 %), G3 (5.4 %) and G2 (4.3 %). Of the VP4 genotypes detected, P[8] was the most prevalent (66.5 %), followed by P[4] (21.9 %) and P[6] (11.2 %). The distribution of G and P genotypes for each year is shown in Table 2. Of the 102 strains with G9 genotypes, the majority (63 %) were associated with P[8] followed by P[6] and P[4]. All 11 G2 genotypes were associated with the P[4] genotype (Table 3).

The most prevalent G–P combination was G1P[8] (21.4 %), followed by G9P[8] (20.6 %) and G4P[8] (13 %). Twenty patients were infected with two G types, 18 G1 + G9 and two G4 + G9, and four with two P types, three P[4] + P[6] and one P[8] + P[6]. All mixed infections with more than one G genotype included G9 strains. One stool sample contained

P[9] genotype and additional testing is being conducted to verify the result. The distribution and frequency of G and P genotype combinations are shown in Table 3.

Representative PCR products of G9 and P[6] genotypes were verified by nucleotide sequencing. The VP7 sequences of 2007, 2008 and 2009 strains isolated during this study showed >99 % similarity to each other, and >97 % similarity to the reference strain AF060487 (US 1205/Indiana/US). Sequences were submitted to GenBank (accession nos HQ018932, HQ018933 and HQ018934). Sequence alignment and phylogenetic analyses of the VP7 gene of G9 strains isolated during this study demonstrated that these strains belong to the VP7 lineage 3 (Fig. 1).

Data on the RV immunization status were available from the Michigan Care Improvement Registry for 372 patients seen during the study period and revealed that only 33 (8.9 %) had received $\geq$ one dose of RV vaccine (RV5 or RV1). Of those, only 9/33 (27.3 %) received three doses of RV5. Only 17 (7.1 %) of the 238 children included in the study had received $\geq$ one dose of RV5 or RV1; 10/17 were infected with G4P[8] strains.

Using the 14-point scoring system, we evaluated the severity of clinical symptoms of RV gastroenteritis in 125 patients who had complete clinical data available. These included 36 patients infected with G1 strains, 9 with G2, 11 with G3, 19 with G4, 40 with G9 and 10 with G1 + G9 strains. The mean scores in each group were 10.56, 9.78, 11.1, 11.3, 10.8 and 12.6, respectively. Differences among means of severity scores were not statistically significant ($P>0.05$); however, groups were unbalanced in sample size.

DISCUSSION

To our knowledge, this is the first report from the USA identifying the G9 genotype as the predominant circulating

Table 2. Distribution and frequency of G and P genotypes of RV detected in Detroit from 2007 to 2009

Genotype	2007		2008		2009		Total <i>n</i> *	% over the 3-year period
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%		
G1	37	43.0	14	41.2	40	29.0	91	35.3
G2	5	5.8	0	0	6	4.3	11	4.3
G3	2	2.3	1	2.9	11	8.0	14	5.4
G4	15	17.4	4	11.8	21	15.2	40	15.5
G9	27	31.4	15	44.1	60	43.5	102	39.5
Total	86	100.0	34	100.0	138	100.0	258	100.0
P[4]	25	32.1	9	26.5	19	14.6	53	21.9
P[6]	6	7.7	6	17.6	15	11.5	27	11.2
P[8]	47	60.2	19	55.9	95	73.1	161	66.5
P[9]	0	0	0	0	1	0.8	1	0.4
Total	78	100.0	34	100.0	130	100.0	242	100.0

*The totals exceed the number of specimens analysed because of mixed infections.

Table 3. Distribution and frequency of G and P genotype combinations of RV detected in Detroit from 2007 to 2009

Genotype*	2007		2008		2009		Total <i>n</i>	% over the 3-year period
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%		
G1P[4]	11	14.1	7	20.6	2	1.6	20	8.4
G1P[8]	18	23.0	7	20.6	26	20.6	51	21.4
G2P[4]	5	6.4	0	0	6	4.8	11	4.6
G3P[8]	2	2.6	1	2.9	10	7.9	13	5.5
G3P[9]	0	0	0	0	1	0.8	1	0.4
G4P[4]	5	6.4	0	0	0	0	5	2.1
G4P[6]	0	0	0	0	2	1.6	2	0.8
G4P[8]	9	11.5	4	11.8	18	14.3	31	13.0
G9P[4]	2	2.6	2	5.9	6	4.8	10	4.2
G9P[6]	6	7.7	6	17.6	9	7.1	21	8.8
G9P[8]	12	15.4	7	20.6	30	23.8	49	20.6
G1P[4 + 6]	0	0	0	0	1	0.8	1	0.4
G1 + 9P[4]	2	2.6	0	0	2	1.6	4	1.7
G1 + 9P[8]	5	6.4	0	0	9	7.1	14	5.9
G4 + 9P[4]	1	1.3	0	0	0	0	1	0.4
G4 + 9P[8]	0	0	0	0	1	0.8	1	0.4
G9P[4 + 6]	0	0	0	0	2	1.6	2	0.8
G9P[6 + 8]	0	0	0	0	1	0.8	1	0.4
Total	78	100.0	34	100.0	126	100.0	238	100.0

*The most prevalent strains of the period are highlighted in bold.

RV genotype after vaccine introduction. Serotype G9 was first detected in the USA in 1983 and later in other parts of the world (Clark *et al.*, 1987). In a Centers for Disease Control and Prevention (CDC) surveillance study of more than 3100 strains from 12 US cities conducted prior to the introduction of RV vaccines (1996–2005), G1 genotype was the most prevalent G genotype in most cities in most years accounting for 50–100 % of tested strains (Gentsch *et al.*, 2009). Detection of G9 strains at the same or higher rate than G1 strains occurred only periodically and in cities submitting low numbers of stool samples, such as in Denver during 1999–2000 when 50 % of 34 samples were G9 and in Atlanta during 2003–2004 when 62 % of 26 submitted stool samples were G9 (Gentsch *et al.*, 2009). In Kansas City during 2002–2003, G9 genotype was detected in 33 % of tested stool samples (Gentsch *et al.*, 2009). Previous studies from the USA and metropolitan Detroit revealed that G9 strains were often detected at low prevalence rates and in association with mixed G genotypes, particularly G1 (Abdel-Haq *et al.*, 2003; Ramachandran *et al.*, 1998). In 2001, our detection rate of G9 infections was 6.3 % and all were components of mixed infections (Abdel-Haq *et al.*, 2003). However, during this study (2007–2009), the overall percentage of G9 infections was almost 40 % and more than 77 % of G9 strains were not associated with other genotypes. Sustained high prevalence of G9 strains suggests that G9 may have established itself as a major genotype in our patient population.

Among the different G–P combinations, the previously uncommon strain G9P[8] was an important cause of RV

diarrhoea in Detroit during the study period. Before the vaccines became available, global rates of G1P[8], G2P[4] and G9P[8] strains were 64.7 %, 12 % and 2.7 %, respectively (Santos & Hoshino, 2005). In our present study, the respective rates were 21.4 %, 4.6 % and 20.6 %. In a review of the global distribution of >21 000 RV serotypes from 35 countries, the four common strains G1P[8], G2P[4], G4P[8] and G3P[8] represented 72 % of all isolates (Gentsch *et al.*, 2005) in contrast to only 44.5 % in our study. In the CDC survey (1996–2005), the most prevalent strain was G1P[8] (78.5 %) (Gentsch *et al.*, 2009). G9P[8] strains accounted for only 3.6 % in the survey but during the current study was the second most common strain from 2007 to 2009. Other recent studies indicate that G9P[8] strains are increasing in prevalence globally (CDC, 2008; de Rougemont *et al.*, 2009; Endara *et al.*, 2007; Esteban *et al.*, 2010; Samajdar *et al.*, 2008). In global surveillance studies conducted by the World Health Organization during the period 2001–2008, the most common genotypes were G1, G2, G3, G4 and G9. Excluding the regions of Africa and the Eastern Mediterranean, where the G and P types of a majority of strains could not be determined, the most common strains in all regions were G1P[8], G9P[8] and G2P[4], accounting for approximately two-thirds of the strains with mean detection rates of 28.3 %, 23.3 % and 16.3 %, respectively (CDC, 2008).

In general, G9 strains have been associated with P[6], P[8], P[11] or P[19] (Araújo *et al.*, 2001; Santos *et al.*, 2001). G9P[6] was the most common genotype combination found with the first emergence of G9 strains in 1995

Fig. 1. Phylogenetic analysis of the VP7 gene nucleotide sequence of human RV G9 strains. The dendrogram was constructed by the UPGMA method. Confidence values based on 100 bootstrap replicates are shown on the branches of the tree. Sequences reported in this study along with the GenBank accession numbers are indicated in the highlighted box.

(Iturriza-Gómara *et al.*, 2000). However, more recent studies suggest that G9P[8] is a more common G–P combination (CDC, 2008; de Rougemont *et al.*, 2009; Esteban *et al.*, 2010). In our patients, G9 serotype was associated with P[8], P[6] and, less frequently, P[4]. P[6] is reported as the third most common P type worldwide and is more prevalent in Africa, Latin America and Asia than in Europe and North America (Gentsch *et al.*, 2005; Steele & Ivanoff, 2003). In our study, P[6] was detected in 27 patients, representing 11.2 % of all P genotypes, and was found in combination with 25 G9 genotypes.

The G9 strains sequenced from the study clustered in G9 VP7 lineage 3 (Fig. 1), consistent with the globally emerging G9 strains. The G9 strains were closely related to the strains isolated in USA during the 1993–2001 RV epidemic seasons (Laird *et al.*, 2003; Ramachandran *et al.*, 2000). G9 (lineage 3) strains include some recently isolated strains from Europe, North and South America, Australia and Asia. Lineage 3 also includes some older Vietnamese and Japanese G9 strains (Ansaldi *et al.*, 2007; Martella *et al.*, 2004; Santos *et al.*, 2005). Nucleotide variations in the lineage 3 of recently isolated G9 strains from various parts of the world ranged from 0.6 % to 2.4 %, whereas it was 3–5 % for the Vietnamese and Japanese strains (Martella *et al.*, 2004).

G9 and P[6] are not included in the current RV approved vaccines. The efficacy of the current RV vaccines against RV types involving G9 is still to be determined. The monovalent vaccine (Rotarix) is based on the concept of heterotypic protection between serotypes caused by cross-reactive antigens while the pentavalent vaccine (RotaTeq) protective efficacy is based on serotype-specific immunity (Gentsch *et al.*, 2005). Despite the fact that G9 is not present in either vaccine formulation, both vaccines have shown protection against G9 strains in previous clinical trials (Ruiz-Palacios *et al.*, 2006; Vesikari *et al.*, 2006). This is probably due to the presence of the P[8] antigen in both vaccines and the fact that most of the G9 strains circulating in the vaccine trial sites were G9P[8]. However, RotaTeq vaccine protection against G9P[6] strains, which share no antigens with the vaccine strains, could be a major challenge. Similar concerns also apply to the Rotarix vaccine. Although this vaccine may provide protection against strains with different G types associated with P[8], this protection may be challenged by strains that belong to genogroups unrelated to the vaccine strain, such as G9P[6] and G2P[4] (Gentsch *et al.*, 2005; Gurgel *et al.*, 2007).

The RV immunization rate of the patients in our study was very low (<10 %). It is likely that this rate reflects the low

immunization rates for RV in Detroit's paediatric population. During June 2006–June 2009, the CDC assessed trends of coverage with ≥ 1 dose of RV vaccine among infants aged 5 months, and compared RV coverage in the second quarter of 2009 with that of DTaP and PCV-7 at eight participating sentinel sites. RV mean coverage was 72% but was 13% lower than mean coverage with ≥ 1 dose of DTaP and PCV-7. In Michigan, the mean site-specific RV coverage was 76%, compared with 92% for DTaP and 91% for PCV-7. However, Detroit was not one of Michigan's sentinel areas (CDC, 2010) and is traditionally lower than most outlying areas in measures of new vaccine coverage.

Although low RV immunization rates may reflect typical new vaccine coverage dynamics (CDC, 2010), the very low rates seen in our patients indicate that significant barriers to routine RV immunization exist. Identification of these barriers and educating families and providers about the benefits of RV immunization will help in reducing severe RV disease.

The severity of RV gastroenteritis did not differ significantly in children infected with different genotypes in our study. Children with mixed infection with G1 and G9 had higher severity scores than those infected with any other single genotype, although this difference was not significant. Cascio *et al.* (2001) have shown that RV strains with a short electropherotype, G2P[4] and subgroup 1 were associated with more severe gastroenteritis symptoms than strains associated with long electropherotypes, G1P[8], G4P[8] and subgroup 2. The authors hypothesized that this association might be due to reintroduction of a G2 serotype, which had not circulated before, in a population that was immunologically naïve to this serotype (Cascio *et al.*, 2001). In a study of hospitalized children in France that was conducted in parallel with emergence of G9 strains, no difference in severity between G1P[8] and G9P[8] infections was found (Aupiais *et al.*, 2009). Similarly, no difference in clinical presentation or severity was found among different G genotypes in another more recent study (de Rougemont *et al.*, 2011). However, correlation between severity and the infecting genotype in our study should be interpreted with caution because of the small and variable number of patients in different groups and the possible bias in only selecting patients hospitalized due to severe disease.

Our results support the need for continuous surveillance of RV strains to monitor the prevalence of G and P types. The increased prevalence of G9 serotypes in our community and its association with different P types, including P[6], raises the possibility that G9 may represent a re-emerging strain that could escape vaccine-induced immunity and become more prevalent following wide use of the new RV vaccines.

In conclusion, there is a diversity of RV strains circulating in the metropolitan Detroit area, including a high proportion of genotype G9 which is not part of the current

vaccine formulations. The majority of our study patients were not RV-vaccinated. However, the protective efficacy of RV vaccines and their effect on the prevalence of G9 strains or on the emergence of other RV strains remain to be determined.

ACKNOWLEDGEMENTS

The study was supported by a grant from Children's Research Center of Michigan, Wayne State University. We thank Dr Marilynn Fairfax for editorial assistance and Dr Ronald Thomas for assistance with statistical analysis. This work was presented in part at the Infectious Diseases Society of America Annual Meeting, Philadelphia, PA, USA, 29 October–1 November 2009.

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