Predominance of genotype P[9]G3 in rotavirus gastroenteritis in Polish children

Anna Piekarska[1], Anna Kacerka[1], Ewa Majda-Stanisławska[1], Barbara Jóźwiak[2], Małgorzata Sidorkiewicz[2]

[1]Department of Infectious Diseases and Hepatology, Medical University of Lodz, Lodz, Poland
[2]Department of Biochemistry, Medical University of Lodz, Lodz, Poland

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Corresponding author:
Anna Piekarska MD, PhD
WSSz Biegański
Building F
1/5 Kniaziewicza St
91-347 Lodz, Poland
Phone: +48 602 358 512
E-mail: annapiekar@op.pl

Abstract

Introduction: Rotavirus (RV) infection is the most common cause of gastroenteritis in children. This paper identifies the most common genotypes of rotaviruses isolated from children hospitalized with gastroenteritis and attempts to determine any relationship between infection with a certain rotavirus genotype.
Material and methods: The investigated group consisted of 68 consecutive children with rotavirus gastroenteritis (confirmed by an agglutination test). Rotavirus genotype was determined in stool samples obtained from each child.
Results: The P[9]VP4 genotype was observed in 41/61 positive samples (over 67.2%) that were permanently associated with the G3 VP7 genotype. Moreover, G3 was determined as the most commonly isolated G type (77.94%). As well as the P[9]G3 type, G3 was also found in the P[4] type (5 cases). Twenty-six out of 61 (42.6%) children in whom rotavirus genotype was determined were co-infected with pathogenic bacteria. No statistical correlation was observed between rotavirus P[9]G3 gastroenteritis and digestive tract co-infection with pathogenic bacteria ($p > 0.05$). Elevated ALT activity was found in 34/59 (57.6%) cases of rotavirus gastroenteritis. Elevated ALT serum level was found to correlate with P[9]G3 rotavirus genotype but concomitant infections did not.
Conclusions: The most common genotype of rotaviruses observed in our group of children, P[9]G3, has rarely been described. Co-infection of the digestive tract with pathogenic bacteria and elevated serum ALT concentrations were found to be the most frequent phenomena. A correlation between P[9]G3 rotavirus genotype and elevated serum ALT level was found, but no significant relationship was identified between concomitant infections and P[9]G3 genotype.

Key words: rotavirus, gastroenteritis, diarrhea, genotype, alanine aminotransferase, bacterial infections.

Introduction

Rotavirus infection is the most common cause of gastroenteritis in children. Almost all children aged 3 months to 5 years become infected [1]. The potential risk of rotavirus infection for a 2-year-old child in Poland was estimated to be up to 5% per year (www. pzh.gov.pl). Rotaviruses are highly contagious and spread as easily as common cold viruses. As with the com-

mon cold, good personal hygiene and drinking water quality do not reduce the risk of infection. Clinical and molecular investigations have confirmed the virulence of rotaviruses: A drop of fluid containing viral particles on a fingertip or on a toy put into the mouth is enough to cause the disease [1–3].

The rotavirus genome consists of 11 fragments of double-stranded RNA, each of which encodes one protein [4]. Structural proteins form a viral particle, while non-structural proteins are necessary for host cell invasion and replication. Structural proteins are termed VP (viral proteins) with consecutive numbers, VP1, VP2 and so on, while non-structural proteins are assigned as non-structural proteins (NSP) with consecutive numbers [5]. Each of 11 segments encodes a different protein, to create 6 structural and 2 non-structural proteins (NSP5 and NSP6). The differences in the structure of the layers of membrane proteins allows them to be differentiated and classified into strains, and for their antigenic response to be determined. The antigenic diversity of outer membrane protein VP6 allows the division of the rotavirus genome into serogroups A to E, sometimes with two additional groups: F and G. Further classification is based on the genetic features and antiserum activity of the viral outer membrane proteins VP4 and VP7. Each of these proteins stimulates production of specific antibodies. While the presence of VP4 protein, which is protease sensitive, determines the P genotype (23 genotypes have been identified), glycoprotein VP7 determines the G genotype (15 genotypes). Ten P genotypes and ten G genotypes have so far been identified in human rotaviruses (HRVs) [5–7].

Investigations conducted previously in Poland reveal that 97% of severe gastroenteritis cases in Poland are caused by four different genotypes: P[8]G1, P[4]G2, P[8]G3, P[8]G4 [8]. In the world, human G1, G3, G4, G9 (commonly associated with P[8]), G2 (regularly associated with P[4]) and G12 (which is predominantly associated with either P[8] or P[6]) rotavirus strains [9] are globally present. Recently, G6 human rotaviruses, mainly associated with P[9] or P[14], have been recovered from children with rotavirus gastroenteritis in Hungary, Italy, Belgium and Australia [9]. G3P[3] and G3P[9] rotavirus strains are believed to be typical feline and canine genotypes [9], Although G2 and G9 were found more frequently in states using Rotarix, whereas G3 was found more frequently in states using RotaTeq, this could be owing to natural genotype fluctuations, as strong fluctuations in the prevalence of different genotype have previously been observed from 1 year to the next or from one state to the other in Australia [9]. In addition, new genotypes such as G9 and G12 emerged and spread worldwide in a very short period of time, suggesting that additional genotypes or new genetic variants of a particular genotype might be able to emerge or spread in the human population when the right conditions appear [9].

Aside from gastrointestinal tract infection, children hospitalized because of rotavirus gastroenteritis frequently present symptoms related to different organs and hepatitis [10]. In addition, an abnormal alanine aminotransferase (ALT) level is frequently observed in children hospitalized in our clinic.

Some authors suggest that the symptoms of hepatitis may accompany an infection with particularly virulent strains [11–13], and it would be interesting to know which kinds of RV strains cause hepatitis, as well as their origin. This paper analyses the most common genotypes of rotaviruses isolated from children hospitalized with gastroenteritis, and attempts to determine any relationship between infection with a particular rotavirus genotype, co-infection with bacteria and elevation of serum ALT.

Material and methods

The investigated group consisted of 68 children hospitalized consecutively between January and March 2010 in the Department of Infectious Diseases and Hepatology, Medical University of Lodz. The reason for hospitalization was always gastroenteritis and vomiting, and a latex agglutination test revealed rotavirus antigen in the stool. A rotavirus genotype was determined in samples obtained from each child. The stool sample for the agglutination test and for genotype determination was obtained during the first day of hospitalization, in the acute phase of the disease. The result of the microbiological stool culture, as well as the serum ALT level, was assessed in each patient.

All children were examined by a physician. Basic laboratory tests including white blood count (WBC), C-reactive protein (CRP) and urinalysis were performed in all patients, while chest X-ray, blood, throat and urine culture were also performed depending on the patient's general symptoms. Each time when elevated serum ALT concentration was found, the panel of viral markers (HBsAg, anti-HBc-total, anti-HAV IgM, anti-HCV) was checked to exclude co-infection.

Patients with abnormal liver enzymes were monitored after they were discharged from the hospital until their blood test results returned to normal levels. Informed consent for the study was obtained from parents of all the children participating in the study.

Genotyping of rotaviruses

Source of RNA

All stool samples were prepared as 20% v/v suspensions in phosphate-buffered saline (PBS),

pH = 7.4. The suspensions were centrifuged for 5 min at 1600 g. The resulting supernatants were used for further analysis.

RNA isolation and cDNA synthesis

Total RNA was isolated from 250 µl of supernatant using TRIrisol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Purified RNA was resuspended in 20 µl of sterile nuclease free water (AppliChem, Darmstadt, Germany). For cDNA synthesis, 2 µg of total RNA and 1 µl of 20 µM P-genotype-specific RT primer (TGG CTT CGC TCA TTT TAT AGA CA) or G-genotype-specific RT primer (TAG CTC CTT TTA ATG TAT GG) (final volume 10 µl) was incubated at 95°C for 5 min, chilled on ice and heated again for 5 min at 65°C. Actual reverse transcription was performed at 42°C for 60 min (final volume 26 µl) using 7 units of Avian Myeloblastosis Virus reverse transcriptase, according to the manufacturer's instructions (Promega, Madison, WI).

P and G genotype determination by PCR

The resulting P- and G-specific cDNAs were used as templates for PCR. For P genotype determination, the following mixture of 20 µM primers was used: P8: TCT ACT TGG ATA ACG TGC. P4: CTA TTG TTA GAG GTT AGA GTC, P6: TGT TGA TTA GTT GGA TTC AA, P9: TGA GAC ATG CAA TTG GAC, P10: ATC ATA GTT AGT AGT CGG. For G genotype determination, the following mixture of 20 µM primers was used: G1: TCT TGT CAA AGC AAA TAA TG, G2: GTT AGA AAT GAT TCT CCA CT, G3: GTC CAG TTG CAG TGT TAG C, G4: GGG TCG ATG GAA AAT TCT, G9: TAT AAA GTC CAT TGC AC.

The reaction (2 min at 95°C followed by 30 cycles of 95°C for 60 s, 50°C for 60 s, 72°C for 60 s plus 72°C for 7 min) was performed with GoTaq-Flexi DNA polymerase (Promega, Madison, WI), according to the manufacturer's instructions.

When no PCR products occurred after PCR, P and G genotypes were determined using nested PCR. In the first step of PCR, cDNA samples were amplified with primers P' (ATT TCG GAC CAT TTA TAA CC) for P typing and G' (GTA TAA AAT ACT TGC CAC CA) for G typing. In the second step of PCR, 5 µl of the first step product was amplified as described above with a mixture of P-or G-specific primers The sequence of primers used in cDNA synthesis, PCR and nested PCR originated from Fischer *et al.* [14].

PA gel analysis

P and G genotype specific PCR products were analyzed in 10% PA gel electrophoresis and visualized with ethidium bromide staining.

Stool sample

Each stool sample was initially examined by the latex agglutination test (immunochromatography) Vikia Rota Adeno (BioMerieux) – stool samples positive for rotaviruses and negative for adenoviruses were considered for further evaluation. Every stool sample was cultured on MacConkey agar, solid SS medium, liquid SF medium, SMAC, Chapman agar and Campylogel.

Serum alanine aminotransferase activity

Serum ALT level was assessed in serum samples obtained from the children with rotavirus gastroenteritis by the kinetic method with NADH and TRIS.

Statistical analysis

Statistical analysis was carried out using the χ^2 test. The statistics package used was Statistica Visual Basic (Stat Soft, Pl).

Results

Serotyping of rotaviruses

Rotaviruses were classified into P types by RT-PCR of the VP4 gene. The expected lengths of P-specific PCR products were as follows: 345 bp for P[8], 483 bp for P[4], 267 bp for P[6], 391 bp for P9 and 583 bp for P[10]. The identification of P-specific PCR products is demonstrated in Figures 1 A–B. G-type classification of rotaviruses was carried out by RT-PCR of the VP7 gene. The expected lengths of G-specific PCR products were as follows: 158 bp for G1, 244 bp for G2, 466 bp for G3, 403 bp for G4 and 110 bp for G9. Identification of the G-specific PCR products is demonstrated in Figures 1 C–D.

A total of 68 samples were included in genotype determination. Most of them were successfully typed with one-step PCR. Remaining specimens (n = 24) that yielded either P or G band as well as specimens that yielded neither of the two bands were reexamined with two-step amplification. Despite using the two-step PCR procedure, neither P nor G type was identified in 7 cases. A summary of genotyping results is presented in Table I.

According to the P and G typing results, P[9] genotype is the predominant P type in the analyzed group of Polish children. The P[9] VP4 genotype was observed in 41 cases (over 67.2%) that were permanently associated with the G3 VP7 genotype. Moreover, G3 was determined as the most commonly isolated G type (77.94%). Apart from the P[9]G3 type, G3 was also associated with the P[4] type (5 cases). P[4] and P[10], the less prevalent P types, were found in 6 and 4 cases, respectively. The P[10] type was exclusively correlated with the presence of the G2 type in samples.

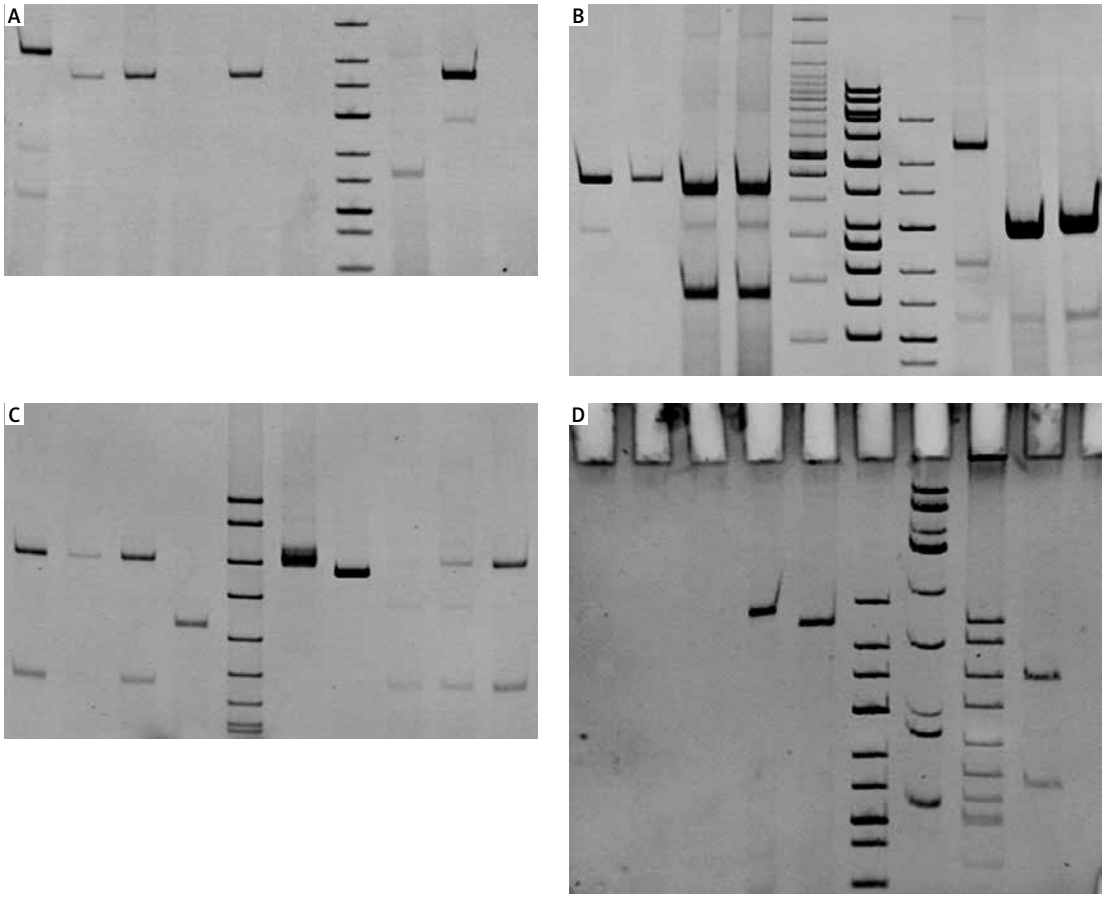

Figure 1. A – G1G3 identification. **B** – G2G3 identification. **C** – P6P9 identification. **D** – P10P9 identification

The P[6] type was established only in 1 sample that was not defined for the G type. Neither the G4 nor the G9 genotype was detected in G typing. None of the P type evaluated samples were assigned to P[8].

Relationship between rotavirus genotype and co-infection with pathogenic bacteria

Twenty-six out of 61 (42.6%) children in whom rotavirus genotype was determined were co-infected with pathogenic bacteria: Genotype P[9] G3 was found in 16/26 (61.5%) of them. Thirty-six out of 61 (57.4%) children in whom rotavirus genotype was determined revealed only physiological bacterial flora in stool samples. Twenty-three out of 35 (65.7%) revealed infection with P[9]G3 genotype. No statistical correlation was found between rotavirus P[9]G3 gastroenteritis and digestive tract co-infection with pathogenic bacteria ($p > 0.05$).

Table I. Summary of the P (VP4) and G (VP7) genotyping of human rotaviruses from children in Poland

Type	P4	P6	P9	P10	ND
G1	1				1
G2				4	1
G3	5		41		7
ND		1			7

ND – Not defined.

Pathogenic bacteria were cultured from 17/26 (65.3%) stool samples, among which there were 6 pathogenic *Escherichia coli* strains, 4 strains of *Enterococcus faecalis*, 3 *Klebsiella pneumoniae*, 2 *Enterobacter cloacae*, 1 case of *Salmonella enteritidis*, and 1 of *Clostridium difficile* infection.

Respiratory tract infections were diagnosed in 12/26 children (46.1%): 7 cases of which were pneumonia while the remaining 5 were upper respiratory tract infections. One child was diagnosed with urinary tract infection, and another with severe purulent dermatitis.

The results of genotyping of rotavirus in children with bacterial co-infection are presented in Table II.

Relationship between viral genotype and elevated serum ALT activity

The ALT activity was assessed in 59 out of 61 children in whom rotavirus genotype was determined. Elevated ALT activity was found in 34/59

(57.6%) cases of rotavirus gastroenteritis (> 31 U/l, range from 32 to 96 U/l, mean 44.5 U/l).

In 25/59 cases (42.4%) ALT activity was within the range of laboratory norms. The correlation between genotyping of rotaviruses and ALT abnormalities is presented in Table III. Twenty out of 34 children (58.8%) with abnormal serum ALT concentration were infected by P[9]G3 genotype. Infection with P[9]G3 was found in 18/25 (72%) children with a normal serum ALT level. A statistically significant difference was found between elevated level of serum ALT and infection with P[9] G3 rotavirus genotype (p = 0.028).

The relationship between rotavirus genotype and ALT serum level abnormalities is presented in Table III.

Discussion

The present study documents the predominance of the P[9]G3 genotype in stool samples collected in Poland in the season 2009/2010. This pair of genotypes was detected in 41 out of 61 positive samples, which is quite a surprising result, as no single pair of genotypes has so far been observed to predominate [15–20]. Moreover, both the P9 and G3 genotype are rarely found in other studies: the G3 genotype is only seen in 1% to 5% of cases [16–18, 20]. Only Ye *et al.* describe the predominance of genotype G3: 32.8% [21].

Our results are inconsistent with current knowledge about the incidence of rotavirus genotypes in humans: The genotype most frequently described in the literature in Europe and Poland was P[8] G9 [22]. According to the literature, G3P[3] and G3P[9] strains have been detected sporadically in humans. High genetic diversity was observed in all the feline, canine and feline/canine-like human rotavirus strains (HRS), in which multiple interspecies transmissions in combination with reassortment events play an important role [23]. Matthijnssens *et al.* recently showed that HRV strains belonging to the Wa-like genogroup have a common origin with porcine rotavirus strains, while those belonging to the DS-1-like genogroup have a common origin with bovine RV (BRV) strains [24]. These findings highlight the need for close simultaneous monitoring of RVs in animals and humans, since animal RVs have shown to be able to cause severe disease in humans [25–27]. In addition, it is currently unpredictable how the available HRV vaccines will perform against this wide variety of unusual animal RV strains infecting humans [23].

Moreover, Wang *et al.* showed that nearly all the 11 gene segments of G3P[9] RVA strains L621 and E2451 might have originated from feline/canine RVAs, and that reassortments may have occurred among these feline/canine RVA strains, before being transmitted to humans. Strains

Table II. Infections accompanying rotavirus diarrhea according to genotype

Variables	G1				Pnd	G2				G3				Pnd	Gnd	All
	P4	P6	P9	P10		P4	P6	P9	P10	P4	P6	P9	P10		P6	
Digestive tract bacterial infectious					1				2	2		10		1	1	17
Respiratory tract infections:									1	3		6		2		12
Pneumonia										1		4				5
Upper respiratory tract infections									1	2		2		2		7
Urinary tract infection												1				1
Skin bacterial infection												1				1

Bacterial co-infections were found in 26 patients with rotavirus diarrhea. In some children more than one kind of co-infection was detected. nd – not defined.

Table III. Elevated serum ALT concentrations according to genotype of rotavirus

Variables	G1				Pnd	G2				Pnd	G3				Pnd	Gnd	All
	P4	P6	P9	P10		P4	P6	P9	P10		P4	P6	P9	P10		P6	
No. of patients with elevated ALT	1	0	0	0	1	0	0	0	0	1	5	0	20	0	5	1	34
No. of patients with normal ALT	0	0	0	0	0	0	0	0	3	0	1	0	18	0	3	0	25
All																	59

nd – Not defined.

L621 and E2451 were detected in diarrheal stool samples collected from a 46-year-old man and a 2-year, 6-month-old male child in China, in 2006 and 2011 [28].

No paper published so far has described such a result of rotavirus genotyping in large group of children. It is for future studies to determine whether P[9]G3 predominance is a new but permanent phenomenon, or if the genotypes are merely changing in consecutive epidemic seasons.

Moreover, the high percentage of children with elevated serum ALT (57%) accompanying rotavirus gastroenteritis also drew our attention. In various papers, the percentage of children with laboratory signs of hepatitis, expressed as increased serum ALT levels, varies from 15% to 37% [1, 29–31], although it is not clear whether this is due to a potential hepatotropism, or induction of proinflammatory cytokines caused by viral infection and resulting in necrosis of hepatocytes. It should be emphasized that in the described group of children, an unusual frequency of hepatitis cases was accompanied by the rare rotavirus genotype P[9] G3. Statistical analysis confirmed the relationship between the genotype and incidence of hepatitis.

Martella *et al.* note that a feline rotavirus G3P[9] carries traces of multiple reassortment events and resembles rare human G3P[9] rotaviruses [32]. This is a very interesting study, and in the context of our observation suggests the transmission of G3P[9] RVs from different animal species to humans. The problem of canine/feline/human RV reassortants demands further whole genome sequence analysis and RNA-RNA hybridization studies [33–35], particularly to enable vaccination against rotavirus infection.

No children in the study had been vaccinated against rotavirus gastroenteritis. However, vaccines registered in Europe (Rotarix and Rotateq) contain P[1] and P[8] genotypes and may not be effective against the P[9] genotype [35]. Unfortunately, the infrequent use of these vaccines in Poland does not allow the impact of vaccination to be estimated on the absence of P[1] and P[8] genotypes among rotavirus gastroenteritis cases in the investigated group of children. No information concerning the antigenic properties of the P genotype against rotavirus gastroenteritis can be seen in current literature. It is known that current vaccines available also in Poland, such as monovalent, two-dosage Rotarix P[8]G1, induce immunity against G1, G2, G3, G4 and G9 [19]. It seems reasonable to investigate the frequency of various genotypes in different epidemic seasons and the impact of popularized vaccination on the incidence of gastroenteritis caused by different rotavirus genotypes.

It should be pointed out that RV RNA was not detected in stool samples in 7 out of 68 investigated cases, in spite of a positive latex agglutination test result. Material for latex agglutination and for PCR was obtained concurrently from the same stool sample, on the first day of hospitalization, and the PCR result may be due to a small amount of viral particles in stool samples [36].

A considerable proportion (42%) of the investigated children were found to be infected with pathogenic bacteria and rotaviruses, most of which were bacterial digestive tract infections. It is not known whether such co-infection is accidental or if bacterial intestinal infection facilitates entry for rotaviruses. A large number of children from the investigated group were infected with pathogenic bacteria and with genotype P[9]G3 rotavirus, but no statistical significance was associated with this phenomenon. Presumably, investigations concerning such correlations require a larger group, but unfortunately the high costs of molecular tests do not allow all hospitalized children to undergo such screening.

In conclusion, the most common genotype of rotaviruses in our group of children, P[9]G3, has rarely been described so far. This antigenic variant may be resistant to antibodies induced by commercial vaccines. Co-infection of the digestive tract with pathogenic bacteria and elevated serum ALT concentrations were found to be the most frequent phenomena. Nevertheless, the statistical correlation of P[9]G3 genotype and serum ALT elevation has been confirmed.

Conflict of interest

The authors declare no conflict of interest.

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