Eur J Clin Microbiol Infect Dis (2010) 29:555–562
DOI 10.1007/s10096-010-0895-1

ARTICLE

Molecular epidemiology of rotaviruses in Bulgaria: annual shift of the predominant genotype

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Received: 29 June 2009 / Accepted: 16 February 2010 / Published online: 10 March 2010

Abstract Rotavirus molecular epidemiology investigations provide important information about the incidence of rotavirus diseases and rotavirus strains in circulation in the prevaccine era. The purpose of this investigation was to study the burden of rotavirus disease, rotavirus strain diversity, and epidemiology specificities of rotavirus infections in Bulgaria. A total of 3,130 stools collected between 2005 and 2008 were tested by immune enzyme tests. G-P genotype identification of rotavirus strains were performed by reverse transcriptase polymerase chain reaction (RT-PCR). Rotavirus etiology was confirmed in 32.4% of the samples tested. Rotaviruses affected predominantly children under 5 years of age (95.5%), with a peak prevalence between the ages of 7 and 36 months. Four of the five globally distributed rotavirus strains (G1P[8], G2P[4], G4P[8], and G9P[8]) constituted 97.7% of all rotavirus strains in circulation. However, annual shifts of predominant rotavirus G-P genotypes were observed from season to season—G4P[8] was predominant in rotavirus season 2004/2005 (56.8%), but was replaced by G9P[8] in 2005/2006 (77.7%), and G2P[4] (41.6%) and G1P[8] (39.5%) in the following two consecutive rotavirus seasons. Year-round circulation of rotaviruses in the country with increased incidence in the winter–spring season and unexpected peaks preceding the rotavirus seasons were observed. Molecular epidemiology data are needed in Bulgaria for health policy makers in order to introduce routine rotavirus vaccination. The monitoring of rotavirus genetic diversity in Bulgaria in the postvaccination period will contribute to a successful rotavirus vaccination program.

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Introduction

Despite the success of the current public health services and hygiene control in water supply and sanitation, acute gastroenteritis remains the second most frequent infectious disease after respiratory infections all over the world [1, 2]. Rotaviruses are the most common etiologic agent of severe infantile diarrhea. Annually, rotaviruses are responsible for 140 million diarrheal episodes and 2 million hospitalizations and approximately 611,000 deaths worldwide, thus, it remains one of the most important challenges in public health [3].

Rotaviruses are nonenveloped, triple-layered icosahedral particles, of ~75 nm in diameter, comprising the genus Rotavirus in the *Reoviridae* family. The viral genome is composed of 11 segments of double-stranded RNA. The VP7 glycoprotein and the VP4 protease-sensitive protein form the outer capsid; these elicit neutralizing antibodies

and form the basis for the dual classification system for rotaviruses into G and P types [4]. To date, 22 G and 30 P types have been reported in humans, mammals, and birds [4–6]. Among these, only 11 G serotypes/genotypes and 12 P serotypes and 15 P genotypes have been found in humans [7–9]. Although the ability of rotavirus genes to segregate independently upon dual infection could, in theory, lead to 132 G-P combinations, five G and P combinations, G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8], are epidemiologically important in human infections [10, 11]. However, geographical and temporal differences exist in the distribution of G and P types and their combinations: P[6] in association with G1, G2, G3, and G9 and G8 in combination with P[8] and P[6] have been shown to be the second most detected P and G type in India and some parts of the African continent, respectively [11–16]. Recent studies have reported the emergence of uncommon G or P types (G5, G6, G10, G11, G12, P[3], P[7], P[9], P[11], and many more) in different countries [10, 11, 17–22]. These uncommon rotavirus G/P genotypes emerge in humans through zoonotic transmission, and reassortment with common human rotavirus strains can potentially lead to the emergence of a pandemic strain [23].

In 2006, two rotavirus vaccines, a monovalent live attenuated G1P[8] vaccine (Rotarix®, GlaxoSmithKline Biologicals) and a pentavalent human-bovine G1–G4,P[8] reassortant vaccine (Rotateq®, Merck and Sanofi Pasteur MSD), were licensed. Two of the largest clinical trials in history showed that these vaccines are safe and highly effective for preventing life-threatening dehydrating rotavirus diarrhea, providing either serotype-specific or cross-reactive protective immune responses [24, 25].

The introduction of rotavirus vaccine into the national immunization programs of many countries have been accompanied by an estimation of the rotavirus disease burden and rotavirus strain diversity and an evaluation of changes over the rotavirus incidence and rotavirus genotypes in circulation in the postlicensure period.

In Bulgaria, early investigations of rotavirus infections were performed by using electron microscopy and polyacrylamide gel electrophoresis, and established a high incidence of rotavirus gastroenteritis in children <7 years of age: 7–11% among sporadic cases, up to 67% during two outbreaks in the Shumen region [26, 27]. The studies also revealed that children <1 year of age were the most affected (70%), and confirmed the winter seasonality of rotaviruses, with a peak in November–January and a ‘shift’ of predominant rotavirus electropherotype from one rotavirus season to another. In a previous study of the molecular epidemiology of rotaviruses in Bulgaria, which included a limited number of rotavirus-positive stool samples (n = 71), a change of the predominant rotavirus genotype during two consecutive rotavirus seasons was observed [28].

The aim of the present study was to study the incidence and the epidemiological features of rotavirus gastroenteritis among children less than 5 years of age in the country over an extended period of time (2005–2008) and to describe the rotavirus strain diversity.

Materials and methods

Patients and stool samples

A total of 3,130 patients hospitalized because of acute gastroenteritis in nine hospitals around the country were enrolled in the 4-year survey (January 2005 to August 2008). Stool samples were collected during the first few hours following hospitalization in sterile feces containers and were stored at +4°C before sending to the laboratory. The investigation presented herein covers three full rotavirus seasons (2005/2006, 2006/2007, and 2007/2008) and an 8-month period (January–August) of the rotavirus season 2004/2005. A rotavirus season was defined as a 12-month period starting from September of a calendar year until the end of August in the following year. Of a total of 3,082 samples, those from whom age was known were grouped into two categories, those from children under 5 years of age (90.5%, n = 2,789; age range 25 days to 60 months) and those form school-aged children, youths and adults (9.5%, n = 293; age range 61 months to 70 years). The age of the remaining 48 patients was unknown. An acute gastroenteritis episode was defined as the appearance of three or more loose/watery stools or forceful vomiting over a 24-h period. A 10% (wt/vol or vol/vol) stool suspension was prepared in RNase-free water. Stool suspensions were vortexed and then clarified by centrifugation for 10 min at 4,000g and the supernatant was stored at +4°C until further processing.

Detection of rotavirus group A antigen

The commercial enzyme immunoassay RIDASCREEN® Rotavirus (R-Biopharm, Darmstadt, Germany) for the qualitative determination of rotaviruses group A was used for stool specimens screening following the manufacturer’s instructions.

Rotavirus G and P determination

RNA extraction

After vortexing and centrifugation at 4,000g, the supernatant was used for viral RNA extraction by TRIzol® LS reagent (Invitrogen, USA) or the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), as described by manu-

facturers. Extracted viral RNA was resuspended in 50 μl RNase-free water and was stored frozen at −70°C.

G and P genotyping

The rotavirus G and P genotypes were identified by using two different protocols. Positive and negative controls were also included. One-step semi-nested multiplex reverse transcriptase polymerase chain reaction (RT-PCR) using SuperScript One-Step RT-PCR with Platinum® Taq (Invitrogen, USA) and one set of primers described by Das et al. and Cunliffe et al. (for G genotypes) and Gentsch et al. (for P genotypes) [13, 29, 30] was used for the period 2005–2007. Since 2007, a random primed reverse transcription step followed by a two-step PCR procedure was implemented where the consensus primers VP7-F/VP7-R and VP4-F/VP4-R were used for the first PCR reactions for G and P typing, respectively, while the second PCR assays were performed by using mixes of one consensus and type-specific primers as follows: for G genotype determination (VP7-R and aBT1/G1, aCT2/G2, mG3/G3, aDT4/G4, aAT8/G8, mG9/G9, G10, G12) [23, 31, 32], and for P genotype detection (VP4-F and 1 T-1D, 2 T-1, 3 T-1, 4 T-1, 5 T-1, P[11]) [30, 33, 34].

Agarose gel electrophoresis

Detection of PCR products were achieved by staining with ethidium bromide following electrophoretic separation on 2% agarose gel in 1× TBE buffer for 1.5 h at 100 V and visualization under UV illumination. As a control for the PCR product lengths, a 50-bp molecular size marker (Invitrogen, USA) was used.

Results

Incidence of rotavirus gastroenteritis cases

During the four rotavirus seasons included in the study, a total of 3,130 fecal specimens were tested for the presence of rotavirus antigen: 164 in 2004/05 (incomplete season), 464 in 2005/06, 1,165 in 2006/07, and 1,337 in 2007/08 (Table 1). The average incidence of rotavirus-positive gastroenteritis was 32.4% (range 27.5–43.1%). A statistically significant reduction in the incidence of rotavirus-positive samples was observed between 2005/2006 and 2006/2007 ($p < 0.001$) and 2006/07 and 2007/2008 ($p < 0.001$).

Distribution of rotavirus G and P genotypes

Of 1,015 rotavirus-positive samples, 912 were further characterized through G and P typing. G and P types were determined from 92.4% (843/912) of the samples, whilst the remaining 7.6% (69/912) of specimens were partly (59 samples) or fully (10 samples) untypeable. A total of 781 of 912 (85.6%) specimens had a single strain and 62 (6.8%) samples contained mixed strains. A total of ten different G combinations and two P-type combinations were identified among the mixed infections, representing the commonly circulating genotypes. The G and P genotype distributions among the 912 rotavirus-positive patients is shown in Table 2.

Among single rotavirus infections, the majority of the cases (97.7%; 763/781) were caused by four of the most common rotavirus strains which have a global distribution: G1P[8], G2P[4], G4P[8], and G9P[8]. The most frequently detected strains were G9P[8], followed by G2P[4], G1P[8], and G4P[8]; however, differences in the distribution and relative incidence of these genotypes were seen year-on-year (Table 2).

Other strains comprised common G and P types but in unusual combinations, which are a result of reassortment events between common human rotavirus strains, were found in 1.8% of samples (14/781). Unusual rotavirus strains, including G8P[4], G9P[9], and G12P[8], were detected in 0.5% of samples (4/781).

Age and monthly distribution of rotavirus diarrhea cases

The age distributional analysis revealed that the highest incidence of rotavirus infection was in infants between the ages of 7 and 24 months (Table 3). A significant incidence of rotavirus diarrhea was also seen in children 2–3 years old (29.8%; 126/423) and in infants under 6 months of age (27.1%; 135/498).

The seasonal distribution of rotavirus cases showed year-round circulation of rotaviruses in Bulgaria, which was unusual for countries with temperate climate (see Fig. 1). The incidence of rotavirus-positive episodes by month showed that the lowest incidence was in the spring–early summer months (May–July; 10–19%) and the highest was in the winter (January–March; 50–68%). A smaller summer peak was also observed in August: 23.5% (12/51) and 38.4% (76/198) in 2006 and 2007, respectively. Furthermore, in 2007, the summer peak was of greater magnitude than the winter peak.

Discussion

This study on the molecular epidemiology of rotaviruses circulating in Bulgaria is the first long-term systematic study of rotavirus strain diversity. The objectives of the study were to evaluate the incidence of rotavirus infections in pediatric gastroenteritis and to provide information on the rotavirus strain diversity circulating in Bulgaria over a 4-year period.

Table 1 Distribution of stool specimens tested and rotavirus-positive samples during the four rotavirus seasons included in the study

Rotavirus season	Stool samples investigated (*n*)	Rotavirus-positive stools (%)	Stools negative for rotaviruses (%)
2004/2005	164	63 (38.4%)	101 (61.6%)
2005/2006	464	200 (43.1%)	264 (56.9%)
2006/2007	1,165	384 (33.0%)	781 (67.0%)
2007/2008	1,337	368 (27.5%)	969 (72.5%)
Total	3,130	1,015 (32.4%)	2,115 (67.6%)

The burden of rotavirus disease

Rotavirus was detected in 32.4% of the samples tested, an incidence which is comparable with the detection rates reported worldwide (29–45%) and in Europe (27–51%) [3, 35].

In Bulgaria, all hospitalizations due to acute gastroenteritis are recorded. According to the hospital discharge data of the Bulgarian Ministry of Health, for the period investigated, a total of 78,426 acute diarrhea cases were registered [36]. Of them, a total of 9,077 cases (incidence rate between 9.5 and 12.7%, depending on the year investigated) were of bacterial origin, while less than 0.2% (n = 122 for the entire period) were with suspected parasitic etiology. The remainder, 87.1–90.3%, accounted for approximately 69,000 diarrheal cases left undiagnosed. Of these, 60–80% of the cases were children under the age of 9 years, which means that, annually, 10,000–14,000 acute gastroenteritis cases of unknown origin are reported in Bulgaria among hospitalized children less than 9 years of

Table 2 Distribution of rotavirus strains according to G-P genotype combination and season of circulation

Genotype	No. of rotavirus strains				Total	
	Rotavirus season					
	2004/2005	2005/2006	2006/2007	2007/2008	No.	%
Single infections, total	38	161	288	294	781	85.6
Common human rotaviruses					763	97.7
G1P[8]	7	16	35	139	197	
G2P[4]	3	1	142	88	234	
G4P[8]	25	7	1	7	40	
G9P[8]	2	136	109	45	292	
Uncommon human reassortants					14	1.8
G1P[4]	0	0	0	5	5	
G2P[8]	1	0	0	5	6	
G4P[4]	0	0	0	1	1	
G9P[4]	0	0	0	2	2	
Unusual rotavirus strains					4	0.5
G8P[4]	0	1	0	0	1	
G9P[9]	0	0	1	1	2	
G12P[8]	0	0	0	1	1	
Mixed infections, total	3	6	17	36	62	6.8
G type	1	6	5	20	32	
P type	2	0	7	11	20	
dual G + dual P type	0	0	5	5	10	
Untyped strains, total	3	8	36	22	69	7.6
G untypeable	0	5	7	10	22	
P untypeable	3	2	27	5	37	
G + P untypeable	0	1	2	7	10	
Total	44	175	341	352	912	100

Table 3 Age distribution of the patients with acute gastroenteritis tested and patients with confirmed rotavirus infection during the four rotavirus seasons

Age (months)	Patients with acute gastroenteritis tested	Rotavirus-positive patients	
		No.	%
0–3	194	48	24.7
4–6	304	87	28.6
7–12	704	294	41.8
13–24	858	351	40.9
25–36	423	126	29.8
37–48	185	31	16.8
49–60	121	29	23.9
> 61	293	45	15.4
Without information	48	4	
Total	3,130	1,015	

age. We established that the incidence rate of rotavirus gastroenteritis varied between 27.5 and 43.1% (average 32.4%), depending on the rotavirus season. From these data, it can be extrapolated that rotavirus infections account for 3,000–4,000 hospitalizations and more than 100,000 mild episodes of gastroenteritis each year in Bulgaria. Nonetheless, a significant diagnostic gap still remains. Investigation of other virological agents of gastroenteritis not currently investigated for in Bulgaria is likely to narrow this gap considerably and allow for a more accurate estimation of the true burden of disease attributable to the different intestinal pathogens.

Although a reduction in the episodes of gastroenteritis attributable to rotaviruses was seen between 2005 and 2008, it is not clear whether the recent introduction of rotavirus testing and sampling for the current study may be a confounding factor, and only continued surveillance will allow the monitoring of this trend in the long term.

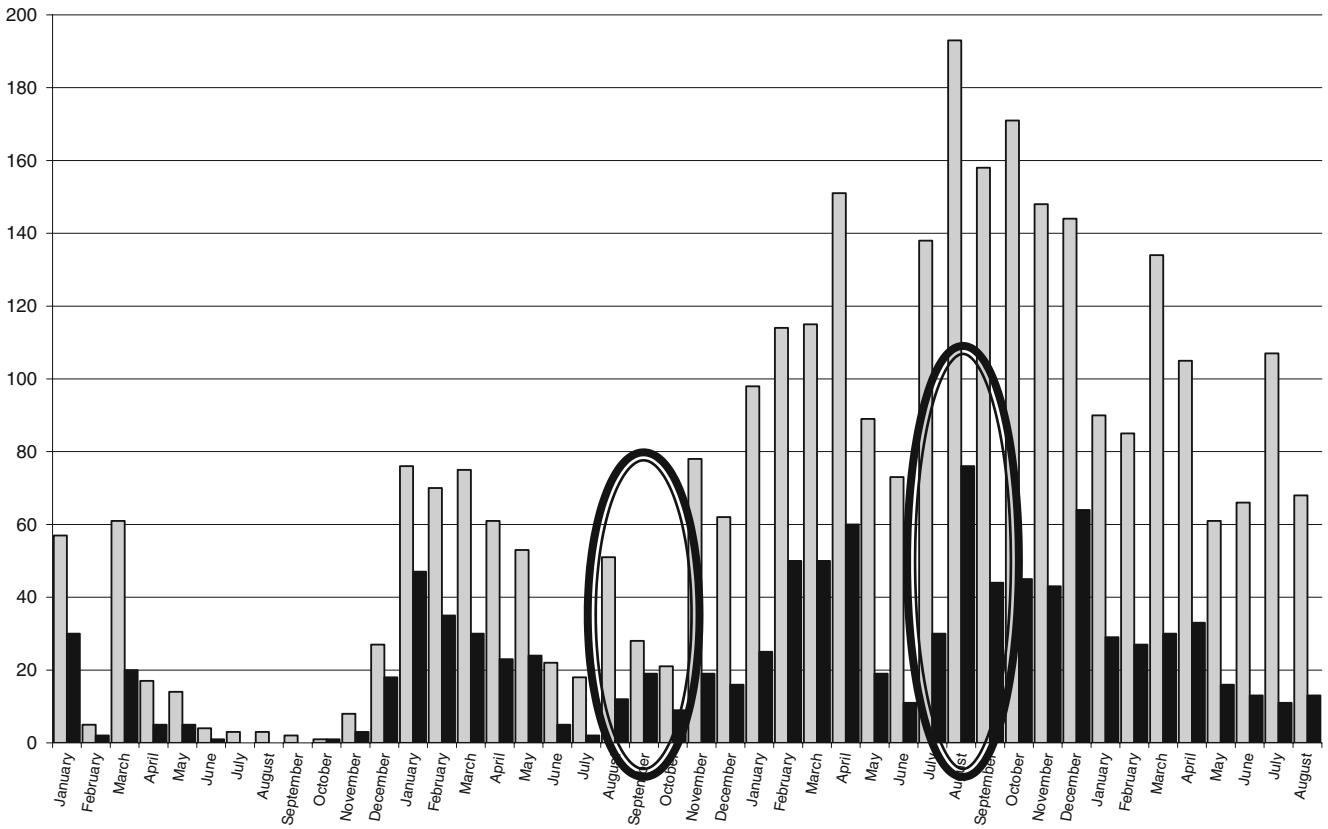

Fig. 1 Monthly distribution of acute gastroenteritis cases tested (*gray bars*) and cases with confirmed rotavirus etiology (*black bars*). The *ovals* delineate the atypical summer peaks of rotavirus gastroenteritis

Rotavirus strain diversity

An interesting observation was the shifts of predominant rotavirus G-P genotypes from season to season. The genotype data analysis has shown that G4P[8] rotaviruses were responsible for more than 65% (25/38) of all hospitalizations during the 2004/2005 rotavirus season, but in the following years, their incidence significantly diminished and remained low until the end of the study period. In the same season, G9P[8] emerged, displacing G4 strains to become the dominant genotype (84.5%). During the following rotavirus seasons, G9 strains remained but with decreasing incidence. G2P[4] strains emerged in the middle of the 2006/2007 season, reaching an incidence of 50%. The incidence of infections with G1P[8] strains was unusually low in Bulgaria at the beginning of the study period, but increased gradually year-on-year and, by 2007/2008, became the predominant genotype. Similar seasonal fluctuations in the distribution of the predominant rotavirus strains are common events and are reported all over the world—in the United Kingdom, Hungary, Slovenia, Ireland, Belgium, Paraguay, Bangladesh, and other countries from southeastern Europe [17, 18, 23, 37–41]. These fluctuations may be driven by herd immunity and the emergence of a particular genotype may reflect the build-up of a sufficiently large population of susceptible children.

Epidemiology of rotavirus infection

The analysis of the epidemiological features of rotavirus gastroenteritis revealed two important observations. First, in the present study conducted in Bulgaria, rotaviruses affected predominantly children under 5 years of age (95.5%), with a peak incidence between the ages of 7 and 36 months (76.3%; 771/1011), in concordance with other countries, where the highest incidence of rotavirus diarrhea was reported in the age group 1–60 months. However, cases of rotavirus diarrhea were identified among older children and adults with an incidence of 15.4%. This is similar to recent reports from the United Kingdom, where rotavirus infections, both symptomatic and asymptomatic, were detected across the age groups [42].

Secondly, we observed year-round circulation of rotaviruses in the country, with increased incidence in the winter season and unexpected peaks preceding the rotavirus seasons. An investigation of the presence of anti-rotavirus group A IgM antibodies in adults conducted in the United Kingdom [43] has shown a high titer of antibodies all year round, despite their seasonal distribution in the child population. Thus, this might suggest the year-round circulation of rotaviruses and an asymptomatic or milder course of rotavirus disease during the summer months, which may be a consequence of herd immunity acquired during the winter months. Furthermore, atypical late-summer peaks of rotavirus gastroenteritis were observed in the investigation, which preceded the newly coming rotavirus season. For instance, in August 2006, the incidence of rotavirus hospitalizations slightly increased up to 23.5%, while in the same month in 2007, it reached 38.4%. This may suggest the introduction of a new rotavirus strain/variant into the immune-naïve population, as previously reported during the emergence of G9 strains in the United Kingdom [23].

In summary, based on the genotype characterization of VP7 and VP4 genes, we established that the infections caused by a single rotavirus strain accounted for 92% of cases. Four of the five common rotavirus G-P combinations, G1P[8], G2P[4], G4P[8], and G9P[8], constituted 97.7% of all rotavirus strains in circulation. Therefore, each of the two licensed rotavirus vaccines should be effective in the prevention of rotavirus disease in Bulgaria. The monovalent Rotarix® (GlaxoSmithKline Biologicals) and the pentavalent human-bovine reassortant vaccine Rotateq® (Merck and Sanofi Pasteur MSD) will provide adequate (heterotypic or homotypic) protection toward the five globally distributed G-P combinations. Moreover, the introduction of rotavirus vaccine into the Bulgarian National Immunization Program, either as mass vaccination or as a recommended vaccine, will significantly decrease rotavirus diarrhea cases among children or associated medical and non-medical expenses. In conclusion, enhanced and long-term investigation of the incidence of rotavirus infections, the changes in rotavirus strain diversity, and of the epidemiological characteristics (variation in regional and temporal distribution) of rotavirus infections in Bulgaria are needed in order to be able to monitor the success of any future vaccination program in Bulgaria.

Acknowledgment This work was partially supported by the European Rotavirus Surveillance Network.

The study was also supported with grants from the World Health Organization (WHO) and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) for the provision of fellowships to ZM in the MRC/MEDUNSA Diarrhoeal Pathogens Research Unit, Medical University of Southern Africa, Pretoria, Republic of South Africa, and the Enteric Virus Unit, Virus Reference Department, Centre for Infections, Health Protection Agency, London, United Kingdom.

The authors are thankful to D. Steele (PATH, USA), J. Gray (HPA, United Kingdom), L. Fiore (Instituto Superiore di Sanita, Italy), F.M. Ruggeri (Instituto Superiore di Sanita, Italy), K. Bányai (Veterinary Medical Research Institute, Hungary), A. Sanchez Fauquier (Instituto de Salud Carlos III, Spain), and Mathew Esona (Division of Viral Diseases, NCIRD, CDC, USA) for their encouragement and help during the entire work.

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