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Genetic characterization of Bulgarian rotavirus isolates and detection of rotavirus variants: challenges for the rotavirus vaccine program?

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Keywords:	rotavirus, genotype, mutant, rotavirus vaccine

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Table 1. Distribution of the four common rotavirus G-P genotype combinations detected during four rotavirus seasons and number of rotavirus strains selected for sequence analysis and described in the study (marked in bold)

GENOTYPE	Total No of ROTAVIRUS STRAINS detected/ No of ROTAVIRUS STRAINS sequenced			
	ROTAVIRUS SEASON			
	2004/2005	2005/2006	2006/2007	2007/2008
G1P[8]	7 / 1	16	35 / 3	139 / 3
G2P[4]	3	1	142 / 2	88 / 3
G4P[8]	25	7 / 1	1	7 / 3
G9P[8]	2	136 / 1	109 / 2	45

Table 2. List of the 19 Bulgarian rotavirus strains selected for analysis. The common amino acid substitutions in different lineages are bolded

Rotavirus strain ID	Year/place of collection	PCR genotype	Sequence genotype	Reference strain	Key aa polymorphism
BG149/2005	May 2005/Sofia-region	G1	lineage I	WA	N94S; D97E ; S146N ; M217T ; I218V
BG1053/2007	July 2007/Sofia	G1	lineage II	WA	D97E ; I218V
BG1120/2007	August 2007/Sofia	G1	lineage I	WA	D97E ; S146N ; L147F; M217T ; I218V
BG1153/2007	August 2007/Sofia	G1	lineage II	WA	D97E ; I218V
BG1670/2007	November 2007/ Sofia	G1	lineage I	WA	T91N; N94S; G96D; D97E ; S146N ; M217T ; I218V
BG45/2008	December 2007/Pleven	G1	lineage II	WA	D97E ; I218V
BG389/2008	May 2008/Sofia	G1	lineage I	WA	D97E ; S146N ; L147F; M217T ; I218V
BG80/2006	January 2006/Sofia	G2		DS-1	A87T ; D96N ; N213D
BG915/2007	June 2007/Sofia	G2		DS-1	A87T ; D96N ; N213D
BG1643/2007	November 2007/Sofia-region	G2		DS-1	A87T ; D96N ; N213D
BG1726/2007	August 2007/Varna	G2		DS-1	A87T ; D96N ; N213D
BG683/2008	August 2008/Sofia-region	G2		DS-1	A87T ; D96N ; N213D
BG170/2006	March 2006/Pleven-region	G4	lineage I	ST3	-76N ; F79S ; R143K ; V145T
BG1385/2007	September 2007/Sofia-region	G4	lineage IX	ST3	D74N; N75D; F79S ; S87T; P90Q; T96N; V145A
BG623/2008	July 2008/Sofia-region	G4	lineage I	ST3	-76N ; F79S ; R143K ; V145T
BG773/2008	September 2008/Sofia-region	G4	lineage I	ST3	-76N ; F79S ; T96N; R143K ; V145A
BG167/2005	October 2005/Sofia	G9	lineage III, sub-lineage d	WI61	A87T ; N100D ; K150E ; T208I ; A220T
BG4/2007	January 2007/Sofia	G9	lineage III, sub-lineage d	WI61	A87T ; G94R; N100D ; K150E ; T208I ; A220T
BG825/2007	June 2007/Sofia-region	G9	lineage III, sub-lineage d	WI61	A87T ; N100D ; K150E ; T208I ; A220T

Figure 1. Phylogenetic tree based on partial VP7 gene (nt 201-771) of G1 rotavirus isolates constructed using Neighbor-joining method of MEGA4. The sequence nomenclatures show the origin, name and laboratory number, and season of circulation (where data are available). Bulgarian G1 strains are marked by the symbol ■. On the right, the clustering of the strains into lineages is represented.

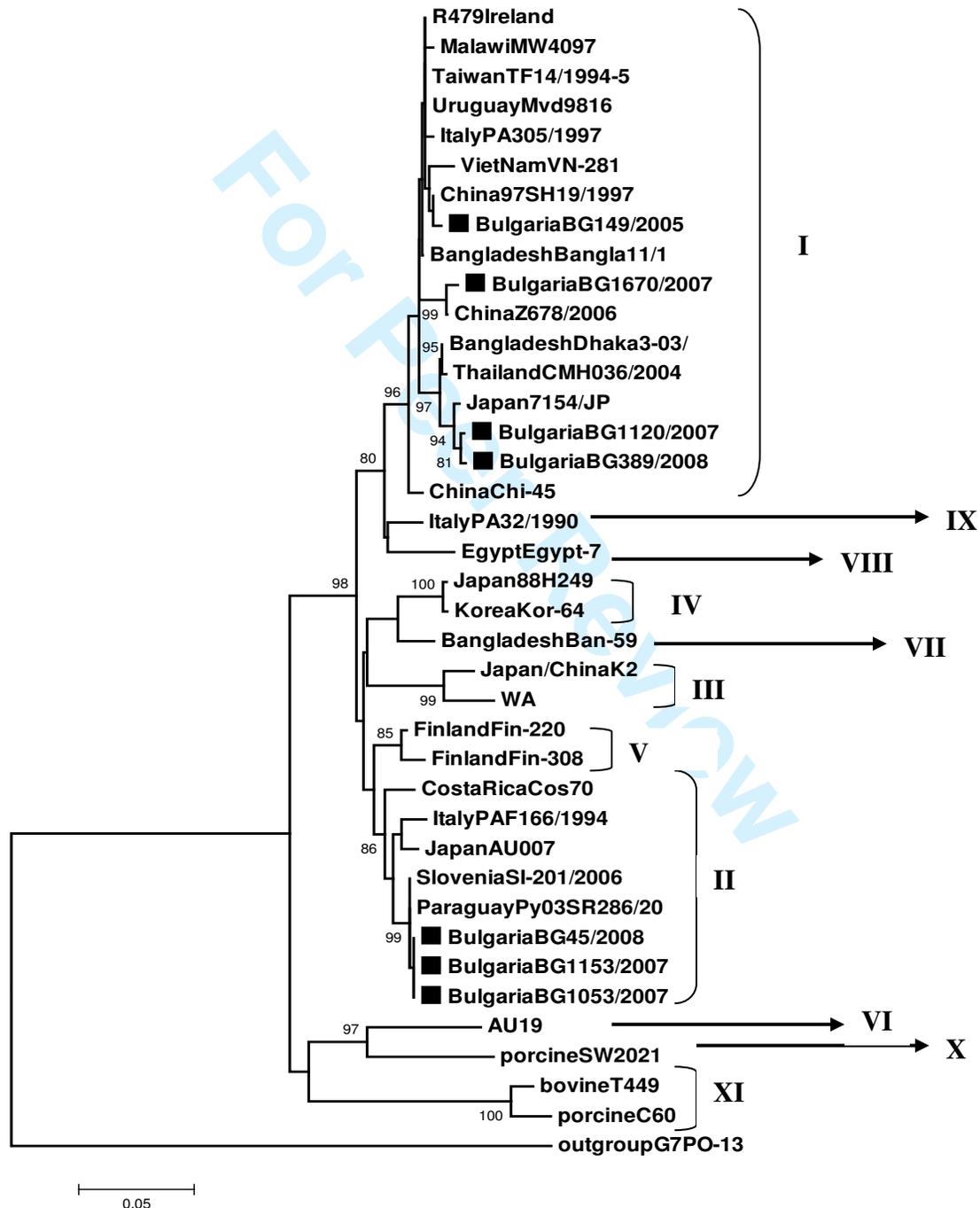
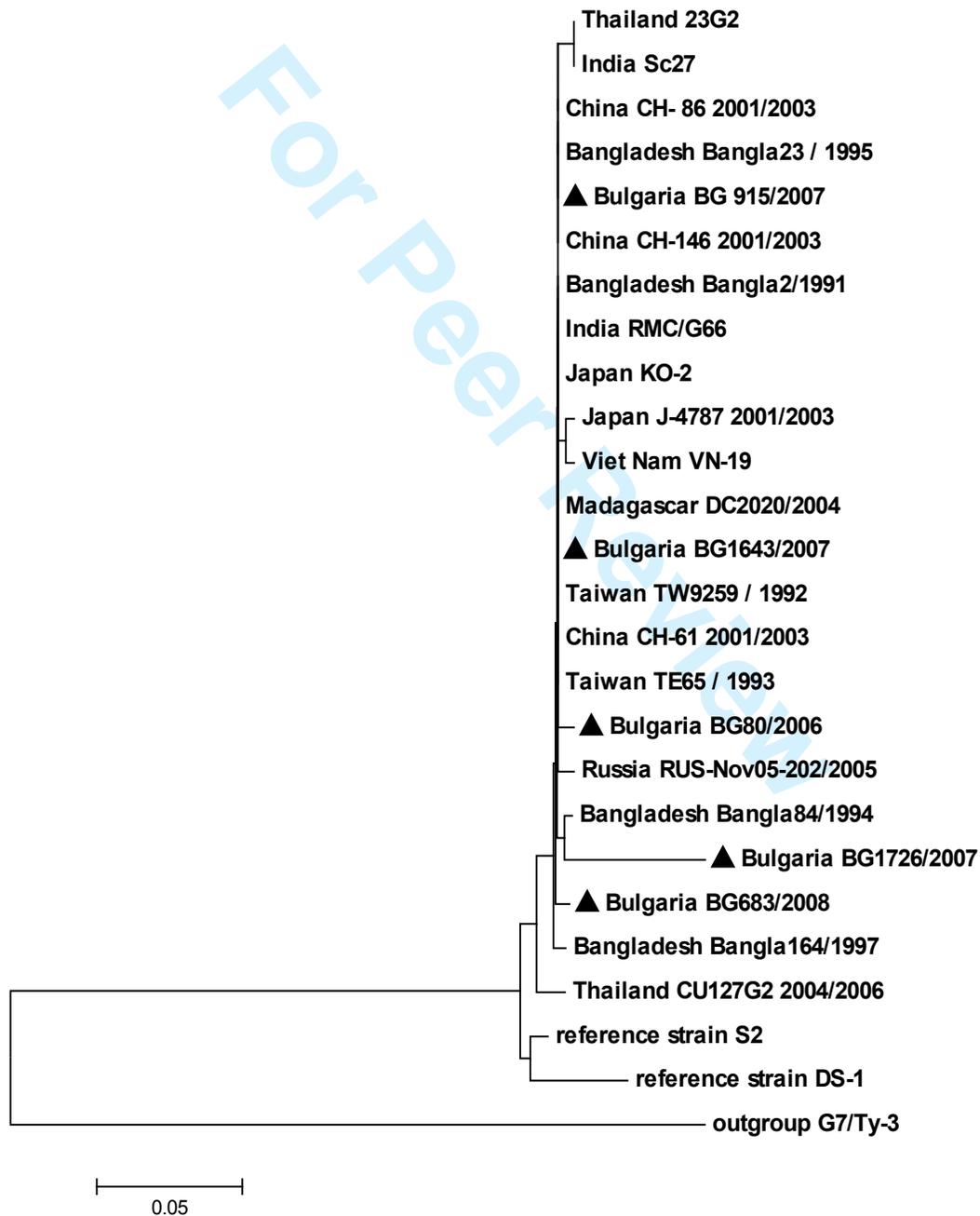
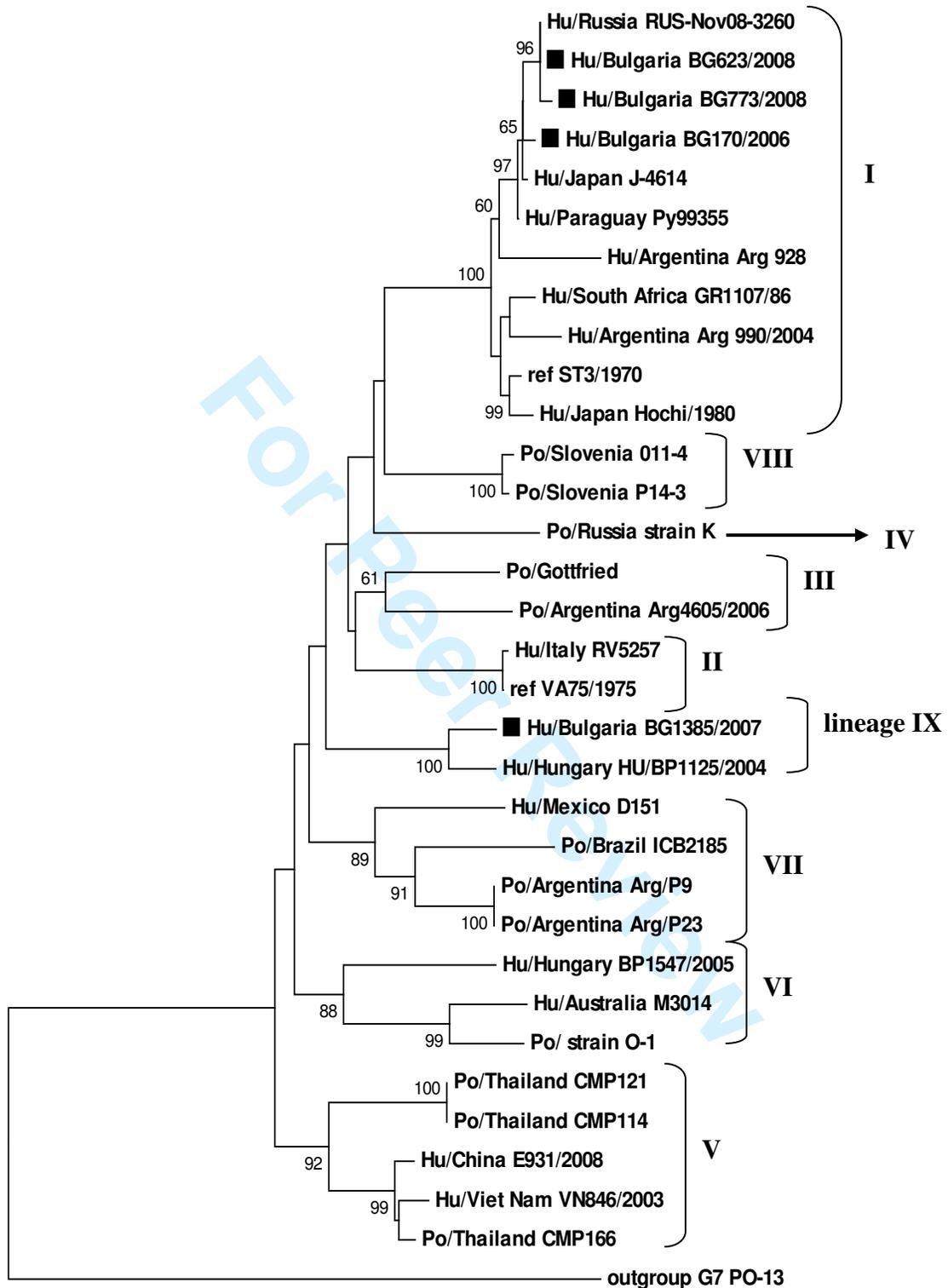


Figure 2. Phylogenetic analysis of the partial nt sequences of VP7 gene (nt 126-717) of rotaviruses with G2 genotype. The phylogenetic tree was constructed based on Neighbor-joining method (MEGA 4 analytical software). Nomenclature of the sequences indicates the origin, name and laboratory number, season of isolation (where possible). The Bulgarian rotavirus strains are indicated by the symbols ▲.



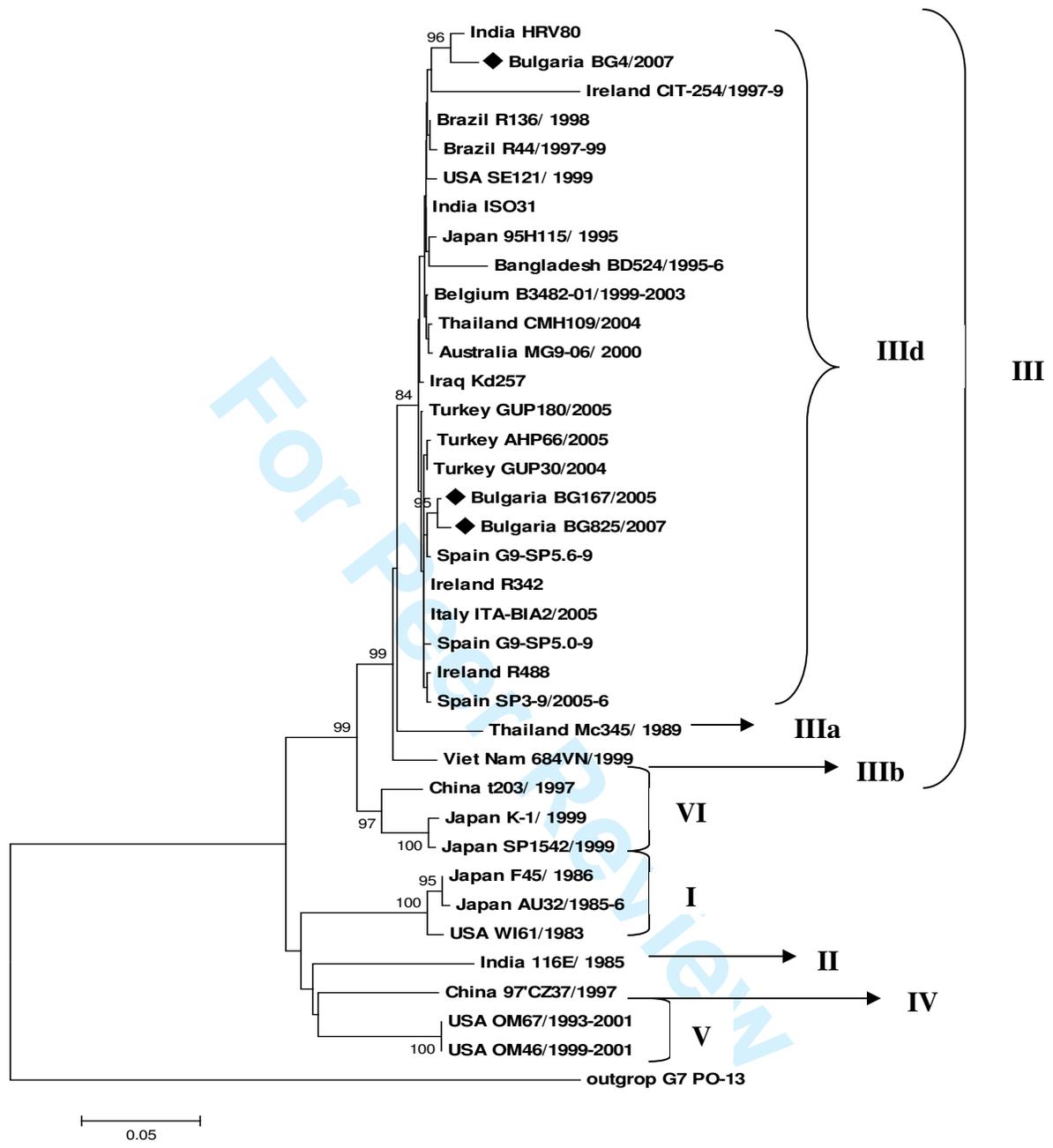
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3 **Figure 3.** Phylogenetic analysis of the partial nt sequences of VP7 gene (nt 178-560) of
4 rotaviruses with G4 genotype. The phylogenetic tree was constructed based on Neighbor-
5 joining method (MEGA 4 analytical software). Nomenclature of the sequences indicates
6 the origin, name and laboratory number, season of isolation (where possible). The
7 Bulgarian rotavirus strains are indicated by the symbols ■. On the right, the clustering of
8 the strains into lineages is represented.
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3 **Figure 4.** Phylogenetic analysis of the partial nt sequences of VP7 gene (nt 165-914) of
4 rotaviruses with G9 genotype. The phylogenetic tree was constructed based on
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6 Maximum-Likelihood method (MEGA 4 analytical software). Nomenclature of the
7
8 sequences indicates the origin, name and laboratory number, season of isolation (where
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10 possible). The Bulgarian rotavirus strains are indicated by the symbol ♦. On the right, the
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12 clustering of the strains into lineages and sub-lineages is represented.
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4 1 **Genetic characterization of Bulgarian rotavirus isolates and detection of**
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6 2 **rotavirus variants: challenges for the rotavirus vaccine program?**
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1 Abstract

2 Annually 20-70% of all hospital admissions and 20% of fatal diarrhea cases among
3 children less than 5 years of age occur due to severe rotavirus diarrhea. Universal
4 immunization is the major strategy aimed at controlling rotavirus infection. The main
5 objective of the present study was to elucidate the evolutionary relationships of the most
6 common rotavirus strains co-circulating in Bulgaria. The sequence and phylogenetic
7 analysis revealed strain diversity and circulation of different rotavirus variants belonging to
8 a single genotype. A mutated G4P[8] strain with the insertion of an asparagine residue in
9 position 76; G2, G9 and G1 variants with amino acid substitutions in the antigenic regions
10 A, B and/or C were all identified in this study in the absence of an immunization program.
11 Rotavirus strain surveillance in both the pre- and post-vaccine eras is of increasing
12 importance in order to assess the effectiveness of the rotavirus vaccines for protection
13 against disease associated with a diverse population of rotavirus strains.

14
15 **Key words:** rotavirus, genotype, mutant, rotavirus vaccine

1 Introduction

2 Human group A rotaviruses have been recognized as the most important etiological agents
3 of severe diarrhea in infants and young children throughout the world. The WHO estimates
4 that annually 20-70% of all hospitalizations and 20% of fatal diarrhea cases in children <5
5 years of age occur due to severe rotavirus infection [Parashar et al.; 2006; Estes and
6 Kapikian, 2007]. Rotavirus vaccination is recognized currently as the best strategy for the
7 global control and prevention of severe rotavirus illnesses in young children, as
8 recommended by WHO. As vaccination becomes used more widely, the knowledge of
9 strain distribution pre- and post-vaccine introduction becomes of increasing importance in
10 order to monitor any possible changes in strain distribution and assess vaccine efficacy
11 against co-circulating and potential emerging rotavirus strains.

12
13 Rotaviruses are classified as a genus within the *Reoviridae* family and contain a genome of
14 11 segments of double-stranded RNA which encode 6 structural (VP1-VP4 and VP6, VP7)
15 and 6 non-structural (NSP1-NSP6) proteins [Estes and Kapikian, 2007]. The VP7 and VP4
16 gene segments code for two outer capsid proteins, a glycoprotein (G) and a protease-
17 sensitive protein (P) that define genotypes/serotypes and form the basis for classifying
18 group A rotaviruses into G and P types [Estes and Kapikian, 2007]. The VP7 and VP4
19 coded proteins elicit neutralizing antibodies after natural rotavirus infection or rotavirus
20 vaccination.

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22 To date, 23 different G genotypes and 32 P genotypes have been described [Matthijnsens
23 et al., 2008; Schumann et al., 2009; Solberg et al., 2009; Trojnar et al., 2009; Ursu et al.,

1 2009; Matthijnsens et al., 2009], indicating extensive genomic diversity within group A
2 rotaviruses. Rotavirus genotypes G1P[8] G2P[4], G3P[8], G4P[8] and G9P[8] are
3 associated most frequently with diarrhea in children. However, other uncommon G and P
4 types (for example G5, G6, G8, G10, G12, P[9], P[11] and P[14]) have been reported in a
5 variety of locations worldwide [Desselberger et al., 2001; Santos and Hoshino, 2005].
6 Some of these genotypes such as G5P[8] in Brazil, G8P[6] in Malawi, and G10P[11] in
7 India are important causes of diarrhea in countries where they are found [Alfieri et al.,
8 1996; Cunliffe et. al., 1999; Iturriza-Gomara et al., 2004], and are likely to emerge by
9 zoonotic introduction. Zoonotic transmission and reassortment with human adapted strains
10 play a major role in the emergence and spread of rotavirus strains in the human population
11 [Iturriza-Gómara et al., 2001; Martella et al., 2010]. Another mechanism important for the
12 generation of strain diversity, which could lead to emergence of antibody escape mutants,
13 is the accumulation of point mutations [Estes and Kapikian, 2007].

14
15 National and international strain surveillance networks have been established worldwide to
16 document the prevalence of local strains and to monitor for possible emergence of
17 uncommon G and P genotypes prior to the introduction of rotavirus vaccination and during
18 the implementation of vaccination programs. In this context, the Bulgarian Rotavirus
19 Surveillance Network was established in order to investigate the rotavirus epidemiology
20 and strain diversity in the country in pre- and post-vaccine eras. In previous studies it was
21 demonstrated high incidence of rotavirus gastroenteritis among hospitalized children less
22 than 5 years of age and co-circulation of common and uncommon rotavirus G-P genotype
23 combinations [Mladenova et al., 2010; Tcheremenskaia et al., 2007]. The objective of the

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1 present study was to provide baseline molecular-genetic data on rotavirus strain diversity in
2 the absence of rotavirus vaccination in Bulgaria and to compare the findings in other
3 countries.

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1 **Materials and methods**

3 **Rotavirus specimens**

4 From January 2005 to August 2008, a total of 1015 rotavirus-positive stool samples were
5 collected from children less than 9 years of age and adults, who presented with acute
6 diarrhea at nine different hospitals in Bulgaria [Mladenova et al., 2010]. After genotyping
7 for the G and P genotypes using primers and protocols described previously [Gouvea et al.,
8 1990; Gentsch et al., 1992; Iturriza-Gomara et al., 2000; Iturriza-Gomara et al., 2001;
9 Iturriza-Gomara et al., 2004; Simmonds et al., 2008], 19 rotavirus strains comprising four
10 G4P[8], five G2P[4], three G9P[8] and seven G1P[8], representing four of the five globally
11 common strains in circulation were selected at random for the present study (Table 1). The
12 rotavirus strains selected for investigation were collected from children under the age of 5
13 years.

15 **VP7 and VP4 characterization by sequence analysis:**

16 The extracted dsRNA of each of the 19 selected strains was denatured at 97°C for 5 min
17 and RT-PCR was carried out using a One- Step RT-PCR kit (Qiagen, Valencia, CA)
18 according to manufacturer's instructions. Forward and reverse primers described
19 previously were used for the amplification of the gene encoding VP7 and the VP8* portion
20 of the VP4 gene segment [Gouvea et al., 1990; Gentsch et al., 1992; Iturriza-Gomara et al.,
21 2000; Iturriza-Gomara et al., 2001; Iturriza-Gomara et al., 2004; Simmonds et al., 2008].
22 After denaturation of the dsRNA, reverse transcription (RT) of each gene from each sample
23 was carried out for 30 min at 50°C, followed by 15 min at 95°C to inactivate the reverse

1 transcriptase and activate the DNA polymerase. The cDNA was then subjected to 30 cycles
2 of PCR in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City,
3 CA) using the following conditions: 60 sec at 94°C; 60 sec at 50°C; 60 sec at 72°C,
4 followed by a single 7 min extension at 72°C and a 4°C cooling step. Amplicons were
5 purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to
6 the manufacturer's protocol. Sequencing of each amplicon was performed with the same
7 consensus primers used for RT-PCR, using a Big Dye Terminator cycle sequencing Ready
8 kit v3.1 (Perkin-Elmer, Applied Biosystems). Automated separation and base-calling of
9 cycle sequencing products was carried out using an ABI 3100 sequencer (Applied
10 Biosystems). The VP7 and VP4 gene derived sequences are freely available from the
11 author upon request.

13 *Phylogenetic analysis*

14 The rotavirus chromatogram sequencing files were edited manually and contigs were
15 prepared using CLC Main Workbench program
16 [<http://www.clcbio.com/index.php?id=1220>]. The rotavirus genotype was confirmed by
17 alignment of each of the Bulgarian sequences with sequences published in the GenBank
18 database using BLAST search [<http://www.ncbi.nlm.nih.gov>]. Dendrograms were
19 constructed after multiple alignments using ClustalX and neighbor-joining or maximum
20 parsimony method listed in the MEGA 4 analytical package [Tamura et al., 2007] and 1000
21 bootstrap replicates were used for statistical evaluation. Bootstrap values of >80 were used
22 to define genetic lineages among clusters of sequences. Genetic distances were calculated

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3 1 by the Kimura two-parameter method. The accession numbers and strains used for this
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6 2 study are shown in Appendix 1.
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1 Results

2 *Genetic diversity among G1 isolates*

3 Phylogenetic analysis was conducted on the following G1 Bulgarian rotaviruses:
4 BG149/2005, BG1053/2007, BG1120/2007, BG1153/2007, BG1670/2007, BG45/2008 and
5 BG389/2008. The phylogenetic tree, based on the classification described by Phan et al.
6 [2007], revealed that Bulgarian G1 rotaviruses belonged to two different lineages (Figure
7 1). Four of the Bulgarian G1 rotaviruses that circulated during different rotavirus seasons -
8 BG149/2005, BG1120/2007, BG1670/2007 and BG389/2008 were grouped within lineage
9 I, together with G1 strains detected worldwide during last ten years. The similarity in
10 nucleotide (nt) and deduced amino acid (aa) sequences of these rotaviruses varied in range
11 of 94-99% and 97-100%, respectively. Based on the deduced aa sequence, strains 97'SH19,
12 CMH026, Dhaka 3-03, ChZ678 and two Bulgarian strains BG149/2005 and BG1670/2007
13 had a substitution at position 94 in antigenic region A, while the Japanese isolate 7154J and
14 Bulgarian strains BG1120/2007 and BG389/2008 possess aa change in antigenic region B
15 (aa 147).

16 The remaining three Bulgarian G1s (BG1053/2007, BG1153/2007 and BG45/2008)
17 belonged to cluster II together with G1 rotaviruses from Europe, Latin America and Asia.
18 These strains showed a similarity level of 99% with an G1 strain from Paraguay
19 (Py03SR286), isolated in 2003 and was also similar to a G1 strain SI-201 detected in
20 Slovenia three years later. The amino acid (aa) substitutions of the Bulgarian and the
21 reference G1 strain WA are shown in Table 2.

1 ***Genetic diversity among G2 strains***

2 A total of five G2 strains, representing different seasons of circulation (including
3 BG80/2006, BG915/2007, BG1643/2007, BG1726/2007, and BG683/2008) were selected
4 for VP7 sequence analysis. The data revealed that the Bulgarian G2 strains belonged to
5 one, highly homogenous cluster (95-99% similarity at the nt and aa levels; Figure 2). The
6 comparison with the reference G2 strain DS-1 showed that all Bulgarian G2 rotaviruses
7 sequenced displayed aa changes, similar to that described for G2 strains isolated since the
8 mid-1990's - two aa changes within antigenic region A (aa 87 and aa 96), and additional aa
9 substitution in position aa 213 in antigenic region C (Table 2).

10

11 ***Genetic diversity among G4 isolates***

12 Four Bulgarian G4 strains (BG170/2006, BG1385/2007, BG623/2008 and BG773/2008)
13 were analyzed through sequencing of the VP7 encoding gene (Figure 3). Strains
14 BG170/2006, BG623/2008 and BG773/2008 clustered in lineage I, together with human
15 strains Py99355, J-4614 and RUS-Nov08-3260 detected in Paraguay, Japan and Russia,
16 respectively. They showed 98-99% and 100% homology at the nt and deduced amino acid
17 (aa) level, respectively. All 6 strains in this cluster possessed an insertion of three nts
18 (ATA) which led to the insertion of a single asparagine residue at position 76 in the aa
19 sequence in relation to prototype strain ST-3 (Table 2). Three additional aa substitutions,
20 one in position 79 close to the antigenic region A and one in the region B (aa 143 and aa
21 145) were also observed.

22 The fourth strain, BG1385/2007, shared 85-86% and 94-97% homology at the nt and aa
23 levels, respectively, with the other three Bulgarian G4 strains described above and other

1 human and animal G4 rotaviruses presented in the GenBank database to date. This strain
2 was closely related to a Hungarian G4 human rotavirus detected in 2004, Hu/BP1125. The
3 phylogenetic analysis indicated that these two strains formed a separate lineage from those
4 previously described, and denominated lineage IX following the classification of Stupka et
5 al., [2009]. The differentiation of the new lineage IX was supported by nt and aa similarity
6 distances (data not shown). Neither G4 strain in this new lineage possessed the insertion at
7 position 76, and differed from the reference strain ST3 in seven aa positions: aa 74, aa 75
8 and aa 79 in close proximity to antigenic region A; three within region A; aa 87, aa 90, and
9 aa 96 and aa 145 in the antigenic region B (Table 2).

11 ***Genetic diversity among G9 isolates***

12 The genes encoding VP7 of three Bulgarian G9 rotaviruses (one from the rotavirus season
13 2005/2006 and two from the season 2006/2007) were sequenced. The Bulgarian G9 strains
14 were closely related to each other with nt identity of 96-98%, but distantly related to G9
15 prototype strains WI61 and 116E isolated from USA and India (85-86% nt similarity).
16 Detailed analysis clearly demonstrated that all Bulgarian G9s belonged to lineage III, sub-
17 lineage d and were genetically similar to G9 strains isolated recently in Europe, Asia,
18 America and Australia (Figure 4). The aa substitutions of the Bulgarian G9s and the
19 reference G9 strain WI61 are shown in Table 2.

21 ***Genetic diversity among P[8] genotype***

22 The partial VP4 gene (VP8*) of nine rotavirus strains with a P[8] genotype, two in
23 combination with G4 VP7 genotype (BG623/2008 and BG777/2008), one with G2

1 genotype (BG683/2008), three with G1 specificity (BG1153/2007; BG45/2008 and
2 BG389/2008) and three G9 rotavirus strains, were sequenced and analyzed. All of the P[8]
3 sequences were clustered in lineage III, except the two G1 strains (BG1153/2007 and
4 BG45/2008) which belonged to WA-like P[8] lineage I (data not shown). The nt homology
5 among strains grouped within lineage III ranged between 96% and 98%.

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1 Discussion

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3 The study described above is the first detailed molecular genetic investigation of rotavirus
4 strain diversity in Bulgaria. Rotavirus surveillance in Bulgaria between January 2005 and
5 August 2008 showed significant morbidity attributable to rotavirus infection in the country,
6 with an incidence of rotavirus diarrhea of between 28 and 43%. Strain characterization
7 showed that 95% of the strains were of the same rotavirus genotypes distributed
8 worldwide, and an annual shift in the predominant rotavirus strain between each of the four
9 rotavirus seasons, defined as starting in September of one calendar year though to August
10 of the following year [Mladenova et al., 2010]. To gain insight into the relationships
11 between the prevalent rotavirus strains in Bulgaria and the common rotaviruses circulating
12 worldwide, 19 previously genotyped rotavirus strains (seven G1P[8] strains, five G2P[4],
13 four G4P[8], and three G9P[8]) collected during these four rotavirus seasons were
14 selected at random and subjected to sequence and phylogenetic analysis of the VP7 gene
15 segment (G genotype). In addition, the partial VP4 gene (VP8*) of 9 rotavirus strains with
16 P[8] genotype (two with G4 genotype, one with G2 genotype, three with G1 specificity,
17 and all three G9 rotavirus strains), were also sequenced. The phylogenetic analyses showed
18 that the Bulgarian rotaviruses with G1, G2, G4 and G9 specificities displayed considerable
19 sequence similarity with the rotavirus lineages of rotavirus strains detected in the last 10
20 years worldwide. Furthermore, the sequence data revealed co-circulation of different
21 rotavirus variants of a single genotype with nt and aa changes in antigenic regions A, B
22 and/or C.

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1 One of the main driving forces of rotavirus evolution is genetic drift. Point mutations are
2 extremely frequent events because rotavirus RNA-dependent RNA polymerase is error-
3 prone with a mutation rate of 5×10^{-5} per nt per replication [Blackhall et al., 1996]. Some
4 point mutations are highly conserved and are inherited by the rotavirus progeny. The most
5 significant are those mutations within the antigenic regions which may lead to the
6 generation of antibody escape mutants. Such antibody escape mutants may lead to the
7 emergence “epidemics” and infect large proportions of the populations causing symptoms
8 even in individuals who may have previously been primed either through naturally
9 acquired wild time rotavirus infections or through vaccination. Furthermore, the emergence
10 and accumulation of nt mutations followed by amino acid transitions in uncommon human-
11 animal reassortants and zoonotic rotavirus strains could drive the adaptation of these strains
12 in the human host and their transformation from sporadic to epidemic/ pandemic strains.
13 The rapid dissemination of G9 rotaviruses in the human population in the early 1990s may
14 have been the result of both genetic shift between animal G9P[6] and human rotavirus
15 strain with P[8] specificity and the continuous adaptation of the newly emerged strain to
16 the human host as a result of genetic drift. Thus, after a decade of sporadic infections with
17 no, or limited, onward transmission, the G9 genotype evolved, acquired a replicative
18 advantage and became one of the five most prevalent human genotypes transmitted
19 worldwide [Santos and Hoshino, 2005].

20
21 In Bulgaria, G4P[8] rotavirus strains were the dominant rotavirus G-P combination
22 (65.8%) in the 2004/2005 rotavirus season, but had low prevalence of 0.3-4.3% throughout
23 the next three seasons until their re-emergence in August 2008. The sequence data provided

1 information that the G4 strains detected in 2005/2006 and the newly-introduced strain in
2 2007/2008 were genetic variants with an aa insertion close to antigenic region A. The first
3 G4P[8] mutants with such insertion were reported in Argentina in 1998, Uruguay in 1999
4 and Brazil from 2000 to 2004. A year later, in 2005, this strain was responsible for a large
5 outbreak of acute gastroenteritis affecting over 64,000 individuals and causing 56 deaths in
6 Nicaragua [Bucardo et al., 2005]. The detailed analysis revealed that this unique insertion
7 together with several other minor mutations led to modification of the secondary structure
8 of the VP7 protein in these human G4 strains, and this change, was probably the trigger for
9 the large outbreak described in Nicaragua in which a high attack rate was observed in
10 adults suggested lack of protection conferred by previous rotavirus infections [Bucardo et
11 al., 2007]. Unfortunately, a G4 isolate, which circulated during 2004/2005 rotavirus season,
12 when the G4 was detected as a dominant strain, was not available for genetic
13 characterization. Sequence analysis of such isolates might have shed some light on whether
14 the predominance of G4P[8] in 2004/2005 was related to the introduction of the strain
15 possessing the insertion or with any other genetic changes which may have also correlated
16 with the emergence of an antibody escape mutant, including the emergence of the novel
17 genetic lineage described in this paper.

18 The G4 rotavirus strain BG1385 showed the closest genetic relatedness with the human
19 rotavirus BP1125/2004 found in Hungary and thought to be of potential zoonotic origin
20 based on its genetic constellation – G4-P[6]-GGII-B, respectively VP7-VP4-VP6-NSP4
21 [Banyai et al., 2009]. The VP7 gene of these two strain showed high degree of similarity of
22 nt and aa similarity (96% and 97% respectively), and only four aa substitution were found
23 between the two strains, all outside the antigenic regions (aa 64, aa82, aa162 and aa 171)..

1 The VP4 associated with the Bulgarian strain was however a P[8], which may indicate that
2 this strain may have undergone reassortment with a human strains, but elucidating its likely
3 origin and evolution will require further work.

4 G2 rotaviruses were the second genotype found most commonly during the four-year study
5 period in Bulgaria. Their incidence throughout the different rotavirus seasons varied from a
6 sporadic spread during seasons 2004/2005 and 2005/2006 to an epidemic scale in
7 2006/2007, with an incidence of 49.3% among patients with confirmed rotavirus diarrhea.

8 The comparison with the reference G2 strains S2 and DS-1 showed that all Bulgarian G2
9 rotaviruses sequenced, displayed two aa changes at positions aa 87 (Ala-Thr) and aa 96
10 (Asp-Asn) in the antigenic region A. The same aa substitution at position aa 96 was
11 reported by Iturriza-Gomara and colleagues [Iturriza-Gómara et al., 2001] who linked it
12 with the failure to react with G2-specific monoclonal antibodies. In another study based on
13 the antigenic and genetic variation of the VP7 gene of human G2 rotaviruses isolated in
14 various African countries, two strains which displayed the same aa change and an
15 additional substitution in antigenic region C, have not reacted with either IC10 or S2-2G10
16 G2-specific monoclonal antibodies [Page and Steele, 2004]. Therefore, the aa substitutions
17 in one or more antigenic regions in the newly emerging G2 rotavirus variant could explain
18 the evasion of pre-existing immunity.

19
20 A total of three G9 and seven G1 strains were investigated in order to characterise
21 Bulgarian G9 and G1 rotaviruses by sequencing of their VP7 genome segments and to
22 compare them with other G9 and G1 rotaviruses circulating in different countries. The
23 results showed the co-circulation of variants in Bulgaria similar to those which have been

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3 1 detected in different parts of the world during the last few years [Banyai et al., 2009;
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5 2 Gentch et al., 2009]. Overall, these data support the likelihood that the introduction of new
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8 3 genetic variants in Bulgaria may be facilitated by current globalization, by transnational
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10 4 and transcontinental transport, trade and migration.
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15 6 In summary, a decision on the introduction of rotavirus vaccine into the routine vaccination
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17 7 schedule in Bulgaria is pending. Rotavirus molecular genetic and epidemiological
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19 8 investigations provide baseline data for the prevalence and strain diversity of rotaviruses in
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21 9 Bulgaria in the pre-vaccine era. Active surveillance and detailed genetic characterization of
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23 10 the common rotavirus strains in the pre-vaccine era are extremely important in order to
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25 11 gather and update information of the currently circulating variants and to identify candidate
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27 12 virus strains suitable for the next generation of rotavirus vaccine. The monitoring of the
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29 13 new variants of the common rotavirus strains in the post-vaccine era, which will have
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31 14 emerged spontaneously or selected under the vaccine pressure, will contribute to assessing
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33 15 their epidemic potential, global spread and impact on the global rotavirus vaccine program.
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3 1 **APPENDIX 1**

4
5 2 **Nucleotide sequence accession numbers**

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7
8 3 Reference rotavirus strains and the accession numbers of their VP7 sequences used in this
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10 4 study were as follow: **for G1 analysis:** 7154/JP (EF079070); Dhaka 3-03 (EF960735);
11
12 5 CMH036 (EF199716); Bangla11 (EF690757); VN-281 (DQ508167); MW4097
13
14 6 (FJ386452); Mvd9816 (AF480293); TF14 (AF183860); PA305/97 (DQ377585); Chi-45
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16 7 (GAU26371); PA32 (DQ377574); Egypt-7 (GAU26373); 88H249 (AB081795); Kor-64
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18 8 (GAU26378); Ban-59 (GAU26366); Fin-220 (Z80294); Fin-308 (Z80297); Cos70
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20 9 (U26370); AU007 (AB081799); PAF166 (DQ377578); Py03SR286 (EF179190); SI-201
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22 10 (EF011972); K2 (D16323); WA (K02033); AU19 (AB018697); R479 (EU033975);
23
24 11 97'SH19 (AF260949); bovine T449 (M92651); porcine C60 (L24164); porcine SW2021
25
26 12 (AF426162); Z678 (EU708572); **for G2 analysis:** S2 (M11164); TE65 (AF106295);
27
28 13 TW9259 (AF044349); RMC/G66 (AY603152); Bangla84 (EF690801); CU12762
29
30 14 (DQ236075); CH-146 (DQ904518); Bangla23 (EF690806); CH-86 (DQ904517); J-4787
31
32 15 (DQ904511); Sc27 (AJ293722); CH-61 (DQ904516); 23G2 (AY660563); KO-2
33
34 16 (AF401754); VN-19 (DQ904513); Bangla2 (EF690807); RUS-Nov05-202 (FJ529394);
35
36 17 DC2020 (FJ436809); DS-1 (EF672581); Bangla164 (EF690805); **for G4 analysis:**
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38 18 Py99355 (DQ015686); J-4614 (DQ904520); RUS-Nov08-3260 (FJ529401); HU/BP1125
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40 19 (AM992548); AgrP/9 (AY115858); Arg928 (AF373918); GR1107 (AF161823); ST3
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42 20 (EF672616); Hochi (AB039035); PV5257 (M86832); VA75 (M86833); Gottfried
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44 21 (X06759); M3014 (X99126); CMP121 (DQ683522); CMP166 (DQ683520); VN846
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46 22 (EF545000); E931 (EU708602); Arg990 (AF373890); BP1547 (AM992555); O-1
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48 23 (AB180972); O11-4 (EU348714); P14-3 (EU348713); Arg4605 (FJ712693); D151
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1 (AJ488586); ICB2185 (AF192267); Arg/P23 (AY115859); CMP114 (DQ683511); K
2 (X58439); *for G9 analysis:* G9-SP5.6-9 (EU15197); AHP66 (AB364375); GUP30
3 (AB364374); G9-SP5.0-9 (EU159192); ITA-BIA2 (EF150330); SP3-9 (EF159955);
4 GUP180 (AB364373); Kd257 (AB247943); ISO31 (DQ117937); R136 (AF438228); CIT-
5 254 (AF281044); R44 (AF438227); SE121 (AJ491192); 95H115 (AB045373); BD524
6 (AJ250543); CMH109 (EF199725); M69-06 (AY307085); B3482-01 (AY487858); Mc345
7 (D38055); 684VN (AB091778); HRV80 (EU483087); R488 (EU033984); 116E (L14072);
8 OM67 (AJ491179); OM46 (AJ491181); 97'CZ37 (AF260959); WI61 (EF672623); F45
9 (AB180970); AU32 (AB045372); t203 (AY003871); K-1 (AB045374); SP1542
10 (AY196109); R342 (EU033981).