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Molecular characterization of rotavirus strains from pre- and post-vaccination periods in a country with low vaccination coverage: The case of Slovenia

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ABSTRACT

Rotavirus vaccination started in Slovenia in 2007 on a voluntarily basis. The vaccination rate is relatively low (up to 27%) and no increasing trend is observed. We present rotavirus genotype distribution among children hospitalized for rotavirus gastroenteritis in Slovenia. Eight consecutive rotavirus seasons were followed, from 2005/06 to 2012/13, and 113 strains of the most common rotavirus genotypes were randomly selected for molecular characterization of rotavirus VP7 and VP4 (VP8*) genome segments. During the vaccine introduction period, from 2007 to 2013, rotavirus genotype prevalences changed, with G1P[8] decreasing from 74.1% to 8.7% between 2007/08 and 2010/11 seasons, replaced by G4P[8] and G2P[4], with up to 52.0% prevalence. Comparable analysis of VP7 and VP8* genome fragments within G1P[8] genotype lineages revealed considerable differences for rotavirus strains circulating before and during the vaccination period. The G1P[8] rotavirus strains from the pre-vaccination period clustered in a phylogenetic tree within Rotarix®-like VP7 and VP8* lineages. However, since 2007, the majority of G1P[8] strains have shifted to distant genetic lineages with lower nucleotide (88.1–94.0% for VP7 and 86.6–91.1% for VP8*) and amino acid (93.8–95.2% for VP7 and 85.3–94.6% for VP8*) identities to the vaccine Rotarix® strain. This change also resulted in a different deduced amino acid profile at the major VP7 and VP8* antigenic epitopes.

1. Introduction

Group A rotaviruses are the leading cause of acute gastroenteritis in children worldwide (Parashar et al., 2006). Although hygiene is a very important preventive measure against the disease, it does not provide an efficient tool for the termination of virus transmission and lowering the disease burden in society. This is evident from comparing morbidity data for developing and industrialized countries. The incidence of rotavirus disease in industrialized countries without rotavirus vaccination is still high, which contributes significantly to the hospitalization rate and physician visits of young children. High direct and indirect costs related to the disease thus justify the development and use of an effective rotavirus vaccine (Parashar et al., 2006).

In rotavirus pathogenesis, the outer capsid glycoprotein VP7 and the protease sensitive protein VP4 have a specific role in the early steps of the virus replication cycle. They express antigenic properties of rotaviruses and possess specific neutralization epitopes (Trask et al., 2012). Neutralizing antibodies can either physically disable virus attachment to the receptor or stabilize the outer layer, thus inhibiting conformational changes needed for rotavirus entry into the cell and uncoating of the outer capsid layer (Aoki et al., 2009). Rotavirus infection can be terminated during these early steps if specific neutralizing antibodies are present.

The genetic characteristics of VP7 and VP4 rotavirus genes are of significant interest for rotavirus molecular epidemiology, determining the dual genotype classification (Gentsch et al., 1992; Gouvea et al., 1990). Genotypes G (for VP7) and genotypes P (for VP4) are assigned to each rotavirus strain based on nucleotide sequence identities. A similar genotyping protocol has also been proposed for other genome segments, comprising the whole genome classification proposed by the Rotavirus Classification Working Group (RCWG) (Matthijnssens et al., 2011, 2008). To date, 27 G and 37 P genotypes have been described, with more than 60 G–P combinations found, of which only 10 have been detected in humans (Matthijnssens and Van Ranst, 2012; Santos and

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Hoshino, 2005). Globally, there are 5 major rotavirus genotype combinations that are the most prevalent in childhood diarrhea: G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] (Banyai et al., 2012). In the 1990s, G12P[8] appeared and became the sixth most common genotype in some geographical areas (Matthijnssens et al., 2010).

Zoonotic potential and interspecies transmission of group A rotaviruses are high, which was already noted in early research steps of rotavirus epidemiology in the 1980s (Cook et al., 2004). However, only a small proportion of zoonotic transmitted strains cause major outbreaks or spread quickly within the human population. Rather, there are sporadic cases with no major impact on the rotavirus epidemiology (Gentsch et al., 2005; Martella et al., 2010; Papp et al., 2013; Steyer et al., 2008). Nevertheless, following rotavirus epidemiology is of high importance, also for detecting these zoonotic strains, since they could undergo genome reassortment and influence the genotype pattern over a longer time scale, as was shown in the case of G9P[8] (Iturriza-Gomara et al., 2000; Matthijnssens et al., 2010).

Following rotavirus molecular epidemiology has been an especially high priority in the last seven years since two rotavirus vaccines, Rotarix® (GlaxoSmithKline) and RotaTeq® (Merck Sharp & Dohme), were introduced and have been in use (Lopman et al., 2012). Some countries have included this vaccine in the national vaccination program and achieved very good vaccination coverage in infants. In those countries, rotavirus incidence and hospitalization rates for rotavirus gastroenteritis have decreased significantly (Buttery et al., 2011; Dennehy, 2012; Msimang et al., 2013; Payne et al., 2013; Standaert et al., 2013; Vesikari et al., 2013). The effectiveness of the two rotavirus vaccines was confirmed in large prelicensure clinical trials and they showed good protection for the most common rotavirus genotypes (Ruiz-Palacios et al., 2006; Vesikari et al., 2006). In the post-licensure period, rotavirus strain surveillance studies are on-going in order to collect epidemiological data on vaccine influence on rotavirus molecular epidemiology and to ensure that the selective pressure of the vaccine will not result in filtration of vaccine escape mutants (Jain et al., 2014; Leite et al., 2008; Lopman et al., 2012; Zeller et al., 2010).

In our study, archived and current circulating rotavirus strains were selected for comparative molecular analysis of the most common genotype strains in Slovenia during eight consecutive rotavirus seasons (2005/06–2012/13). Phylogenetic clustering and a detailed analysis of amino acid residues at the antigenic epitopes were carried out to explain genotype variation after the vaccine introduction period in Slovenia.

2. Materials and methods

2.1. Sample collection

Stool samples were collected mainly from hospitalized children presenting with acute gastroenteritis. Unfortunately, clinical data were not available for most of the children since this was a retrospective study and archived stool samples were analyzed. Stool samples from confirmed rotavirus infections were collected in regional laboratories from six of eight healthcare regions in Slovenia and sent to the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana. Stool samples were stored at −20 °C prior to genotyping and molecular analysis of rotavirus strains. In the study period of the 8 consecutive rotavirus seasons, from 2005/06 to 2012/13, a total of 3227 rotavirus strains were analyzed.

A 10% stool suspension was prepared in 0.2 M PBS (pH value 7.4) and centrifuged for 5 min at 1600×*g* for clarification. An aliquot of 200 µl supernatant was used further for total nucleic acid extraction with an iPrep™ PureLink® Virus Kit (Life Technologies, Invitrogen Division, Carlsbad, CA). Extracted nucleic acid was stored at −80 °C until further use for genotyping.

2.2. RT-PCR and genotyping

For rotavirus genotyping, VP7 and VP8* segments were initially amplified, using VP7-F/R and VP4-F/R primer pairs, adopted from the EuroRotaNet guidelines (www.eurorota.net) (Iturriza-Gomara et al., 2011). Amplification was carried out using the Superscript II One-step RT-PCR system (Life Technologies, Invitrogen Division). The RT and PCR conditions were set up according to the manufacturer's instructions, except for the annealing temperatures, which were 52 °C for VP7 and 50 °C for VP8*, as proposed by the EuroRotaNet guidelines. For genotyping, multiplex PCR was used separately for genotypes G (for the VP7 segment) and genotypes P (for the VP8* segment). For VP7, G1–G4, G8–G10, G12 and for VP4, P[4], P[6], P[8], P[9], P[10], P[11], type specific primers were included in a multiplex reaction (Iturriza-Gomara et al., 2011), using the *Tfi* polymerase enzyme system (Life Technologies, Invitrogen Division). Genotypes were confirmed based on the PCR amplified fragments, separated by 1.5% agarose gel electrophoresis.

2.3. Sequencing and NA analysis

The period of our study included rotavirus consecutive seasons 2005/06–2012/13. In each season, up to 20 rotavirus strains were selected with a representative number of genotypes: G1P[8], G2P[4], G4P[8] and G9P[8]. However, there were not enough strains in each season to reach this target for some genotypes. In total, 113 strains were analyzed: 31 G1P[8] (2 in 2005, 4 in each of 2006, 2007 and 2009–2012 and 5 in 2008); 21 G2P[4] (3 in 2007, 6 in each of 2008 and 2011, 1 in 2009, 2 in 2010, 3 in 2012); 33 G4P[8] (5 in each of 2005 and 2009, 4 in each of 2006, 2008 and 2010–2012, 3 in 2007), 28 G9P[8] (6 in 2005, 5 in 2012, 4 in 2006, 3 in each of 2007, 2009 and 2010, 2 in each of 2008 and 201).

For each rotavirus strain, VP7 and VP8* amplified fragments were purified using the Exo I/FastAP™ enzyme system (Thermo Fischer Scientific, Waltham, MA) (Werle et al., 1994). All genome fragments were directly sequenced with the initial PCR primers used in the first round RT-PCR of the VP7 and VP8* amplification reaction. An ABI PRISM BigDyeTM 3.1 terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) was used for the sequencing reaction in a 20 µl reaction and purified with a BigDye® Xterminator™ purification kit (Applied Biosystems). Sequence data were generated in an ABI 3500 Genetic Analyzer (Applied Biosystems).

Nucleotide sequences were aligned and assembled in CLC Main Workbench 6.9 software (CLC Bio, Aarhus, Denmark). Alignment and the construction of a Neighbor-Joining phylogenetic tree was performed in the MEGA 5.2 software package (Tamura et al., 2011).

Nucleotide and amino acid sequences obtained in this study were deposited in GenBank under accession numbers KJ432637–KJ432862.

2.4. Rotavirus disease burden and vaccination coverage in Slovenia

Epidemiological data of rotavirus infections were collected for children in the age group of 0–6 years for each of the analyzed season to estimate the eventual reduction of rotavirus disease. The overall rotavirus incidence was calculated based on the total reported cases of rotavirus infections (hospitalized and outpatients) and the hospitalization incidence was calculated according to the reported hospitalized cases of rotavirus diarrhea. The incidences were calculated for the total population of children in the age group 0–6 years.

Rotavirus vaccination coverage in Slovenian children was calculated based on the total number of life births in a specific year, divided by the number of fully vaccinated children in the same year.

Official data were obtained from the Statistical Office of the Republic of Slovenia (http://www.stat.si/eng/index.asp) and National Institute of Public Health of the Republic of Slovenia (http://www.ivz.si/).

3. Results

3.1. Rotavirus genotype prevalence and vaccination coverage

In the 2005/06–2012/13 rotavirus seasons, genotypes G1P[8], G2P[4], G4P[8] and G9P[8] represented 84.2–96.2% of all genotype strains detected in our study. Genotype G3P[8] was detected rarely, the highest prevalence was noted in 2006/07 and 2012/13

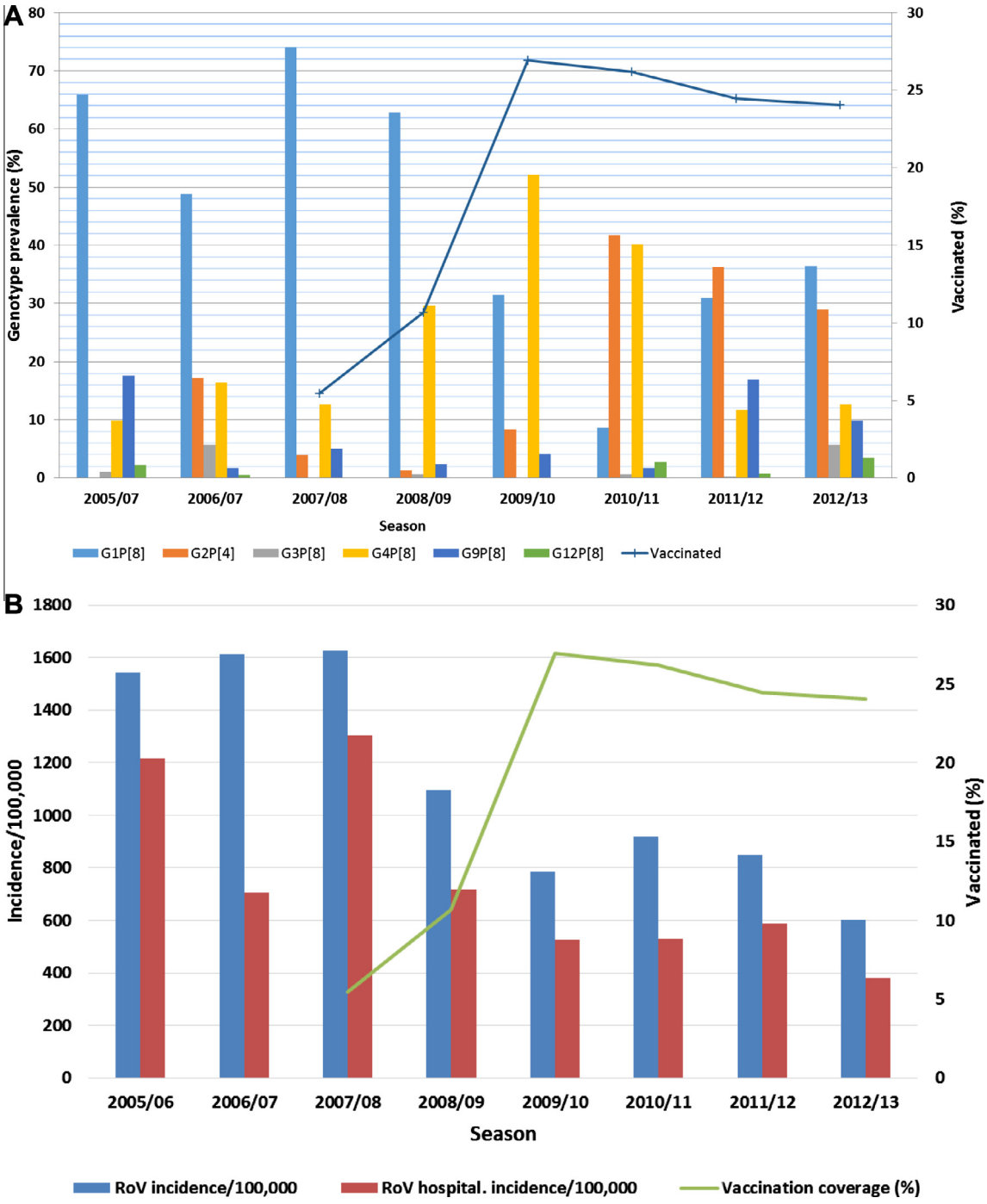

Fig. 1. (A) Prevalence of rotavirus G–P genotypes throughout the study period of eight consecutive seasons 2005/06–2012/13 and the dynamic of vaccination coverage among children 0–1 year of age in Slovenia; (B) presentation of rotavirus disease burden in Slovenia, showing the incidence of rotavirus cases and the incidence of hospitalized rotavirus cases per 100,000 children in the age group of 0–6 years.

seasons with 5.7% and did not exceed a 1% prevalence in other seasons of our study period. Similarly, G12P[8] was detected in up to 3.5% (Fig. 1A).

Although the most prevalent genotypes were as expected, G1P[8], G2P[4], G4P[8] and G9P[8], the genotype prevalence profile changed drastically over the study period. G1P[8] was the most prevalent genotype in the 2005/06–2008/09 seasons. However, G4P[8] increased slightly in the 2008/09 season (29.7%) but presented as the most common genotype in the next season, 2009/10, with 52.1% and G1P[8] was found only in 31.6%. Both prevalences, for G1P[8] and G4P[8], decreased in 2010/11 and, in contrast, G2P[4] became the most prevalent type detected in 41.6% of tested strains and also remained high in the 2011/12 season (36.2%) (Fig. 1A).

Plotting the vaccination coverage in Slovenia to the genotype prevalence graph, a correlation of a sharp decrease of G1P[8] prevalence and increase of vaccination coverage was observed. Vaccination started in Slovenia in January 2007 but only Rotarix® was available in this year and the coverage was 5.5% among children 0–1 years of age. RotaTeq® was introduced only at the end

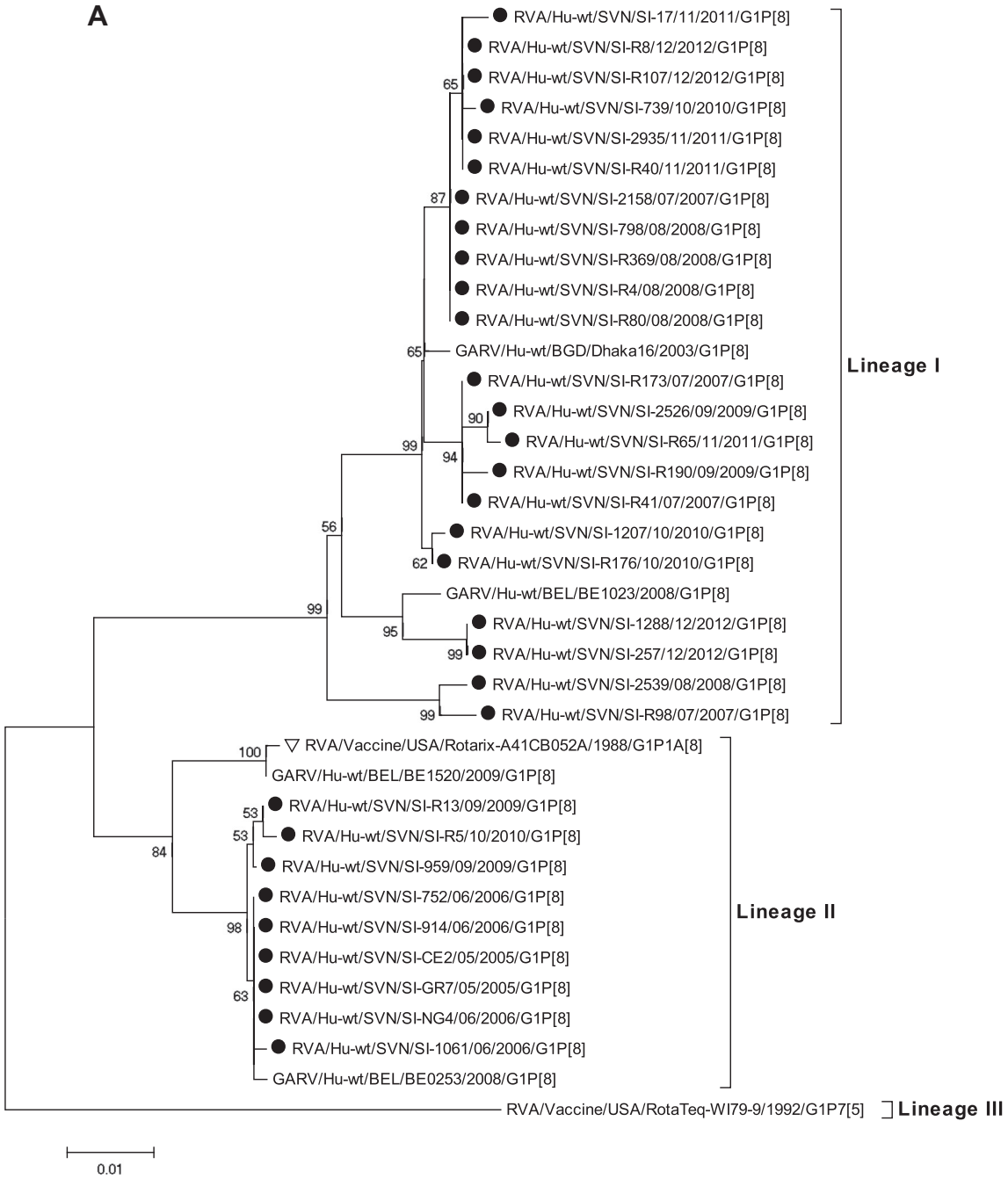

Fig. 2. Phylogenetic analysis of the VP7 nucleotide sequence of Slovenian rotavirus strains, Rotarix and RotaTeq vaccine strains and lineage-specific strains obtained from GenBank (A) G1 genotype VP7 sequences; (B) G2 genotype VP7 sequences; (C) G4 genotype VP7 sequences; (D) G9 genotype VP7 sequences. Neighbor-joining phylogenetic trees were constructed with the Mega 5.2 software package, using the Kimura-2 parameter method with bootstrapping of 1000 replicates. SVN – a unique 3-letter country code for Slovenia.

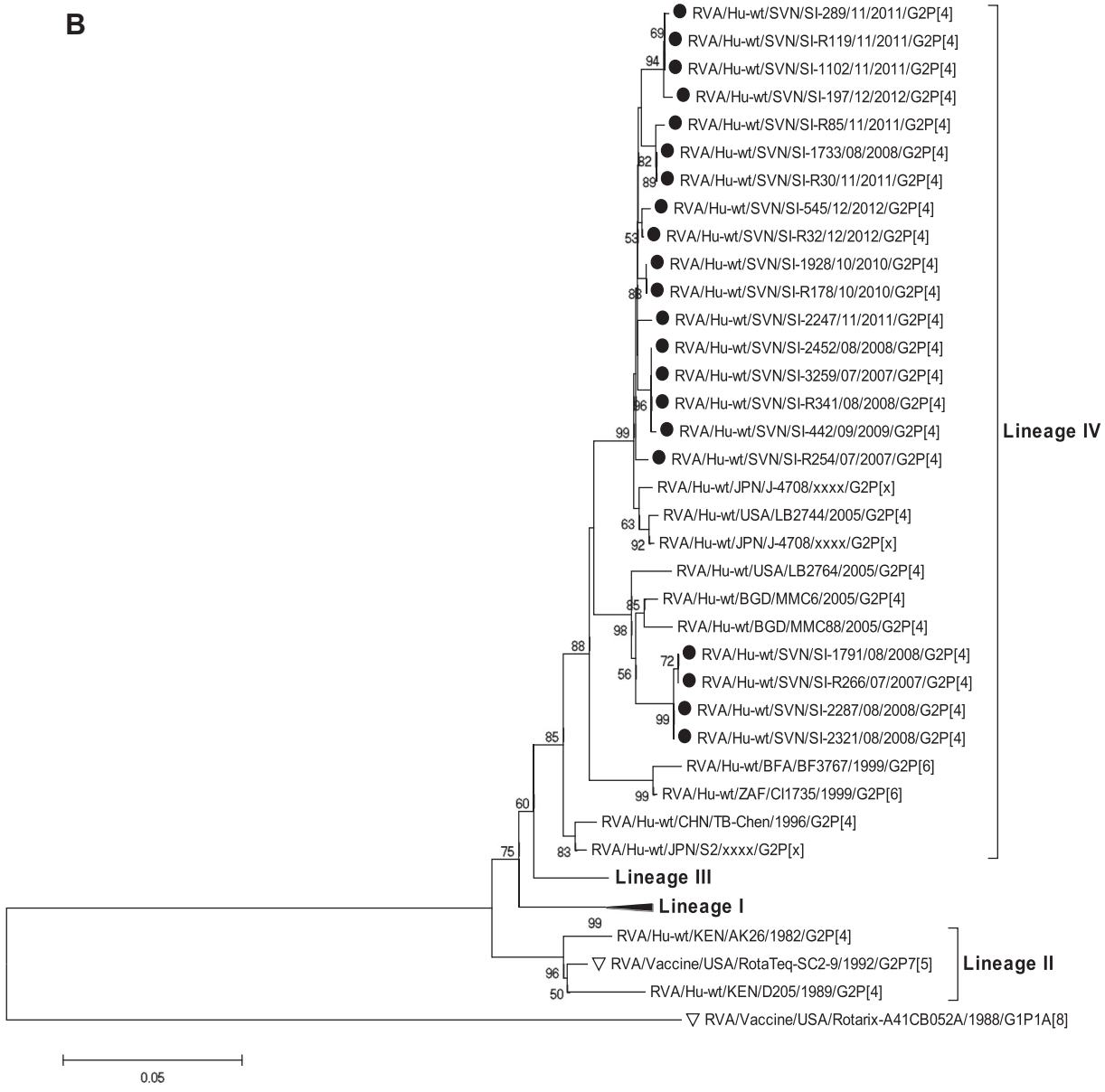

Fig. 2 (*continued*)

of 2008. The highest vaccination coverage was reached in 2009, with 26.9%, and decreased slowly each year, down to 24.5% in 2011 and 24.1% in 2012 (Fig. 1A).

3.2. Rotavirus disease burden

The rotavirus incidence among children 0–6 years was between 1542.01/100,000 and 1627.76/100,000 for the seasons 2005/06 to 2007/08. After this period, the incidence started to decline from 1093.94/100,000 to as low as 603.20/100,000 (Fig. 1B). The reduction was noted also for the incidence of rotavirus hospitalizations among children 0–6 years, ranging between 705.43/100,000 in 2006/07 and 1303.29/100,000 in 2007/08, followed by the decreased level from 717.08/100,000 in 2008/09 to 37.42/100,000 in 2012/13. Both data showed the reduction trend of rotavirus disease burden together with increasing vaccine coverage in the target population.

3.3. Molecular characteristics of rotavirus strains in VP7 and VP4 (VP8*)

For this study, G1P[8], G2P[4], G4P[8] and G9P[8] rotavirus strains were selected for detailed molecular analysis of the outermost proteins, glycoprotein VP7 and the VP8* fragment of VP4, both of them carrying the serotype-specific antigen epitopes and neutralization sites determined in previous studies (Aoki et al., 2009; Dormitzer et al., 2002).

VP7 of G1P[8] strains. As shown in Fig. 2A, the VP7 phylogenetic analysis of G1 rotavirus strains revealed two separate groups. The first group clusters into lineage II, in which the Rotarix® G1P[8] vaccine strain was also found. However, with a bootstrap of 84, Slovenian strains grouped into a different branch than the vaccine strain. The nucleotide sequence identity between the Rotarix® vaccine strain and Slovenian strains within lineage II was in the range of 97.5–97.9% (Table 1). Comparing the deduced amino acid sequences, this identity was 97.3–97.8%. Slovenian G1 strains

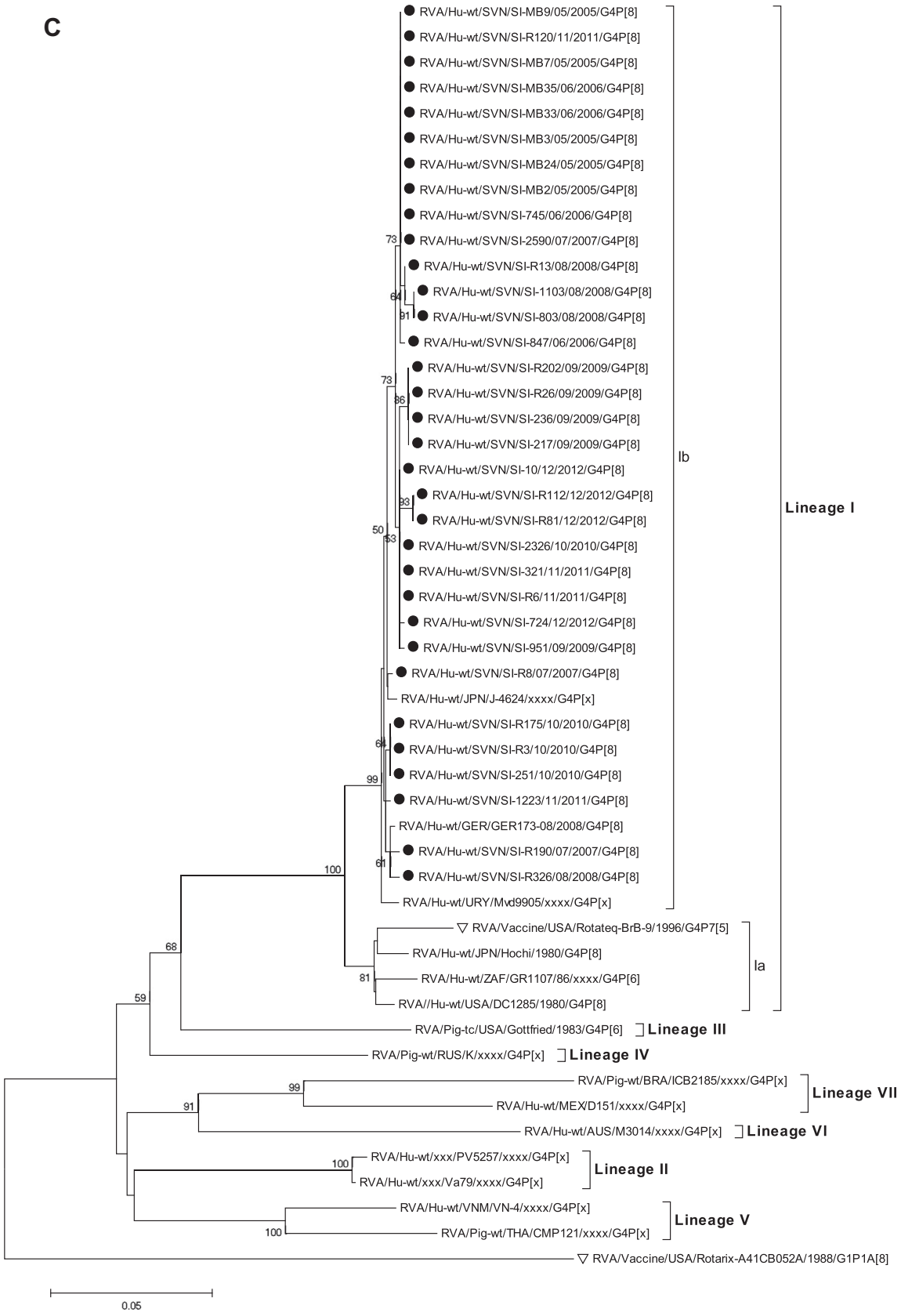

Fig. 2 (*continued*)

within lineage II of VP7 are very homologous, with high nucleotide and amino acid identities (99.4–100.0% and 99.1–100.0%, respectively). Lineage I of Slovenian G1 strains is more variable. Within lineage I, VP7 nucleotide and deduced amino acid sequence identities of Slovenian strains were 90.9–100.0% and 91.6–100.0%, respectively. Rotavirus strains from the post-vaccination period

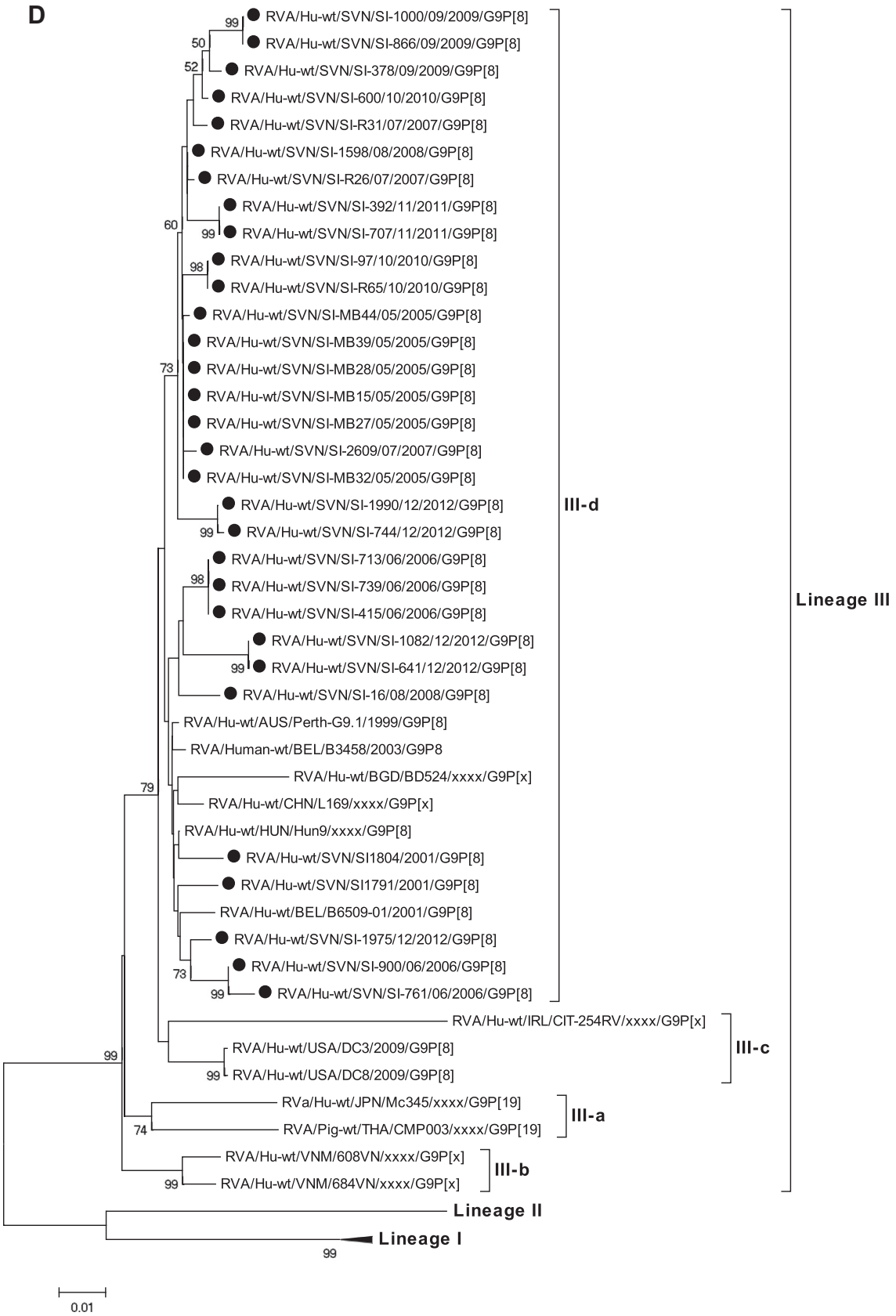

Fig. 2 (*continued*)

were found mostly within lineage I; only three strains were found in lineage II. Interestingly, pre-vaccination strains from the 2005 and 2006 period were clustered exclusively into lineage II. In addition to phylogenetic analysis, deduced VP7 amino acid sequences of G1 rotavirus strains were analyzed and compared to both vaccine G1 strains and selected Slovenian strains of

lineages I and II. The RotaTeq® sequence of the G1 VP7 strain is a representative of the separate lineage III, very distantly related to VP7 lineages I and II of Slovenian G1 strains (Fig. 2A and Table 1).

As shown in Fig. 3, clear changes in amino acid residues of VP7 glycoprotein were noted, previously shown to be of importance for virus neutralization. Representative rotavirus strains from the pre-vaccination period (2005–2006), SI-GR7/05 and SI-1061/06, were identical to the Rotarix® G1 vaccine strain in all amino acid residues within antigenic epitopes 7-1a and 7-1b. Only one amino acid substitution (N147S) was observed in epitope 7-2, which was identical to the RotaTeq® strain and not to the Rotarix® strain. However, amino acid residues of the strains from the post-vaccination period showed differences in all three antigenic epitopes.

VP7 of G4P[8] strains. In our study, G4 rotavirus strains were the most homologous in terms of the molecular characteristics of the VP7 and VP8* fragment of genome segments. All G4 strains were grouped together in VP7 lineage I, sublineage Ib, with 98.4–100.0% nucleotide sequence identity (Fig. 2C). RotaTeq® G4 vaccine strain is slightly different to Slovenian G4 strains, with 94.2–94.8% of VP7 nucleotide and 95.3–96.2% of amino acid identity (Table 1). Aligning the amino acid residues of the VP7 antigenic epitopes to the RotaTeq® vaccine strain, at least four differences were noted, at positions 130, 211, 143 and 145, distributed in all three structurally defined antigenic epitopes 7-1a, 7-1b and 7-2. Only strains SI-R326/08 and SI-951/09 had additional substitutions, at positions 238 and 190, respectively (Fig. 3).

VP7 of G9P[8] strains. G9 rotavirus strains were grouped into VP7 lineage III but forming two clusters (Fig. 2D). Both Slovenian (SVN) major and minor G9 VP7 clusters contain rotavirus strains from the pre- and post-vaccination period. Nucleotide sequence identities within the clusters were 97.2–100% and between clusters 96.5–98.6%. The VP7 genome segment of G9P[8] strains was compared to the strains SI1791 and SI1804 detected in 2001 in Slovenia (Steyer et al., 2005). Identities of these two strains to other Slovenian G9 strains ranged between 97.1–98.4% for nucleotide and 97.1–99.5% for amino acid sequences. Within the antigenic epitopes, a few changes, including positions 100, 148 and 211, were noted comparing 2001 strains to the strains from 2005 to 2012 (Fig. 3).

VP8 of G1P[8], G4P[8] and G9P[8] strains.* Slovenian strains with the P[8] VP4 genotype are grouped for the most part in P[8] lineage III in the phylogenetic analysis (Fig. 4A). G1, G4 and G9 genotype strains were found in this lineage. A small group of analyzed strains was also found in lineage I, together with the Rotarix® G1P[8] vaccine strain. Interestingly, all of them are of G1P[8] genotype combination and the majority of them were detected in the pre-vaccination period. Only three of them were from the 2009/10 season. The same clustering was observed in G1 VP7 phylogenetics. Similarly to VP7, Slovenian rotavirus strains within VP8* lineage I were very homologous, sharing 99.5–100.0% nucleotide and 99.3–100.0% amino acid identities, and 97.5% of nucleotide and 98.0–98.6% of amino acid identities with the vaccine Rotarix® strain. The variability within lineage III was much higher, ranging from 84.2 to 100.0% of nucleotide identity (84.0–100.0% amino acid) (Table 1). The high similarity of VP8* lineage I rotavirus strains to the Rotarix® vaccine strain was also reflected in the antigenic epitopes, in which only one difference at position 113 (region 8–3) was observed between them. In contrast, Slovenian VP8* strains within lineage III were more closely related to RotaTeq® than to the Rotarix® vaccine strain (Fig. 5).

VP7 and VP8 of G2P[4] strains.* All analyzed G2 rotavirus strains were clustered in VP7 lineage IV, as shown by the phylogenetic analysis (Fig. 2B). They share only 92.8–93.9% of nucleotide and 94.6–96.1% of amino acid identity with the vaccine RotaTeq® G2 rotavirus strain, which is found in lineage II. Within

Table 1
Nucleotide and amino acid sequence identities for intra-lineage comparison of the analyzed Slovenian rotavirus genotypes G1, G2 and G4 of VP7 gene and P[8] of VP4 gene. In addition, identities comparing Slovenian rotavirus genotypes to the vaccine strains are presented.

	Rotarix (% identity)				RotaTeq (% identity)								Intra-lineage identities (%)	
	VP7-G1		VP8*-P[8]		VP7-G1		VP7-G2		VP7-G4		VP8*-P[8]			
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
VP7-G1														
Lineage I	88.1–94.0	93.8–95.6			84.6–90.7	91.6–92.9							90.9–100.0	91.6–100.0
Lineage II	97.5–97.9	97.3–97.8			89.8–91.8	94.2–95.1							99.4–100.0	99.1–1000
VP7-G2														
Lineage IV							92.8–93.9	94.6–96.1					95.5–100.0	97.3–100.0
VP7-G4														
Lineage Ib									94.2–94.8	95.3–96.2			98.4–100.0	98.7–100.0
VP8* P[8]														
Lineage I			97.5	98.0–98.6							89.8	91.3–92.0	99.5–100.0	99.3–100.0
Lineage III			86.4–91.1	84.0–90.6							86.9–94.6	85.3–94.6	84.2–100.0	84.0–100.0

	7-1a														7-1b						7-2									
	87	91	94	96	97	98	99	100	104	123	125	129	130	291	201	211	212	213	238	242	143	145	146	147	148	190	217	221	264	
Rotarix G1P[8]	T	T	N	G	E	W	K	D	Q	S	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	M	N	G	
Rotateq G1P[8]	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	
SI-GR7/05	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	
SI-1061/06	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	
SI-2158/07	.	.	S	.	.	.	.	.	.	N	.	.	.	-	.	.	T	.	.	.	.	.	.	.	.	.	T	.	.	
SI-R41/07	.	.	S	.	.	.	.	.	.	N	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	
SI-R98/07	.	N	S	.	.	.	.	.	.	N	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	
SI-R173/07	.	.	S	.	.	.	.	.	.	N	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	
SI-798/08	.	.	S	.	.	.	.	.	.	N	.	.	.	-	.	.	T	.	.	.	.	.	.	.	.	.	T	.	.	
SI-2539/08	.	N	S	.	.	.	.	.	.	N	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	T	D	.	
SI-R190/09	.	.	S	.	.	.	.	.	.	N	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	
SI-959/09	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	S	.	.	I	.	.	
SI-739/10	.	.	S	.	.	.	.	.	.	N	.	.	.	-	.	.	T	.	.	.	.	.	.	H	.	.	T	.	.	
SI-R65/11	.	.	S	.	.	.	.	.	.	N	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	
SI-17/11	.	.	S	.	.	.	.	.	.	N	.	I	.	-	.	.	T	.	.	.	.	.	.	.	.	.	T	.	.	
SI-257/12	.	.	S	.	.	.	.	.	.	N	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	L	T	.	.	
SI-R107/12	.	.	S	.	.	.	.	.	.	N	.	.	.	-	.	.	T	.	.	.	.	.	.	.	.	.	T	.	.	

Rotateq G2P[5]	A	N	S	D	E	W	E	N	Q	D	T	M	N	K	Q	D	V	S	N	S	R	D	N	T	S	D	I	S	G
SI-3259/07	T	.	.	N	.	.	.	.	.	.	.	.	.	-	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	
SI-R266/07	T	.	.	N	.	.	.	.	.	.	.	.	.	-	.	.	.	D	.	N	.	.	.	.	.	.	.	.	.	
SI-1791/08	T	.	.	N	.	.	.	.	.	.	.	.	.	-	.	.	.	D	.	N	.	.	.	.	.	.	.	.	.	
SI-1733/08	T	.	.	N	.	.	.	.	.	.	.	.	.	-	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	
SI-442/09	T	.	.	N	.	.	.	.	.	.	.	.	.	-	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	
SI-1928/10	T	.	.	N	.	.	.	.	.	.	.	.	.	-	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	
SI-R119/11	T	.	.	N	.	.	.	.	.	.	.	.	.	-	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	
SI-197/12	T	.	.	N	.	.	.	.	.	.	.	.	.	-	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	

Rotateq G4P[5]	S	T	S	T	E	W	K	D	Q	N	L	I	D	K	Q	D	T	A	D	T	R	A	S	G	E	S	T	S	G
SI-MB2/05	.	.	.	.	.	.	.	.	.	.	.	.	E	-	.	N	.	.	.	.	K	T	.	.	.	.	.	.	.	
SI-MB35/06	.	.	.	.	.	.	.	.	.	.	.	.	E	-	.	N	.	.	.	.	K	T	.	.	.	.	.	.	.	
SI-R190/07	.	.	.	.	.	.	.	.	.	.	.	.	E	-	.	N	.	.	.	.	K	T	.	.	.	.	.	.	.	
SI-R326/08	.	.	.	.	.	.	.	.	.	.	.	.	E	-	.	N	.	.	E	.	K	T	.	.	.	.	.	.	.	
SI-R13/08	.	.	.	.	.	.	.	.	.	.	.	.	E	-	.	N	.	.	.	.	K	T	.	.	.	.	.	.	.	
SI-R202/09	.	.	.	.	.	.	.	.	.	.	.	.	E	-	.	N	.	.	.	.	K	T	.	.	.	.	.	.	.	
SI-951/09	.	.	.	.	.	.	.	.	.	.	.	.	E	-	.	N	.	.	.	.	K	T	.	.	.	L	.	.	.	
SI-R175/10	.	.	.	.	.	.	.	.	.	.	.	.	E	-	.	N	.	.	.	.	K	T	.	.	.	.	.	.	.	
SI-R120/11	.	.	.	.	.	.	.	.	.	.	.	.	E	-	.	N	.	.	.	.	K	T	.	.	.	.	.	.	.	
SI-724/12	.	.	.	.	.	.	.	.	.	.	.	.	E	-	.	N	.	.	.	.	K	T	.	.	.	.	.	.	.	

SI1791/01 SVN G9	T	T	G	T	E	W	K	D	Q	D	A	I	D	N	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
SI1804/01	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
SI-MB44/05	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	
SI-MB39/05	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
SI-761/06	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
SI-16/08	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
SI-392/11	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	
SI-1990/12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
SI-1082/12	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	

Fig. 3. Deduced amino acid sequence in the antigenic epitopes of the VP7 glycoprotein. At position 291 the information on amino acid residue is missing in most of the sequences presented due to the short nucleotide sequence (marked as (-)).

Slovenian G2 strains, VP7 nucleotide and amino acid identity was 95.5–100.0% and 97.3–100.0%, respectively (Table 1). All of the analyzed strains had three differences in neutralization epitopes in comparison to the vaccine G2 RotaTeq® strain (Fig. 3), two of them within the antigenic epitope 7-1a (A87T and D96 N) and one within the antigenic epitope 7-1b (S213D). Within lineage IV of the VP7 phylogenetic analysis, four strains (SI-1791/08, SI-R266/07, SI-2287/08, SI-2321/08) were grouped separately and all of them also express an additional amino acid difference within the antigenic epitope 7-1b (S242N) (Figs. 2B, 3). These four strains were also grouped separately in the VP8* phylogenetic analysis and present a slightly different pattern of amino acid residues in the antigenic epitopes of VP8*, with one amino acid difference from all other Slovenian G2P[8] strains (VP8* S113I) (Fig. 5).

4. Discussion

Many studies have been published since the introduction of rotavirus vaccines, reporting rotavirus molecular epidemiology in the post-vaccination period. These reports were highly encouraging, especially from countries with a general vaccination strategy, where vaccination coverage within a few years exceeded 80 or 90% of children eligible for vaccination. At this time, it became clear that the rotavirus vaccine was achieving its primary goal of significantly decreasing the incidence of rotavirus gastroenteritis and severe cases of diarrhea. In Europe, a few countries introduced rotavirus vaccination with full or partial reimbursement of the vaccine cost and have a very high vaccination coverage. This is the case in Finland, Belgium and Austria. In Finland, Rotarix® was available from May 2006, RotaTeq® a year later and by 2009, vaccination coverage had reached 35% of the vaccine eligible population, rising to 96% after the introduction of universal vaccination exclusively with RotaTeq® in 2009 (Rasanen et al., 2011; Vesikari et al., 2013). Severe rotavirus gastroenteritis with hospitalization was prevented by vaccine in 92.1% of vaccine eligible children and was reduced by 78% in children <16 years. Despite the high vaccination coverage, no significant changes in rotavirus genotypes was observed to be connected directly to the high selective

pressure of the vaccine (Vesikari et al., 2013). However, with detailed molecular analysis of circulating strains during the period of Rotarix® use, a shift of the P[8] lineage, with multiple changes at the dominant antigenic epitopes, was observed, which might be connected to the use of Rotarix® vaccine with up to 30% vaccine coverage at that time (Hemming and Vesikari, 2013). In Belgium, with an average vaccination coverage of 88.5% with Rotarix® vaccine (Zeller et al., 2010), a significantly higher relative prevalence of G2P[4] was observed in vaccinated children compared to unvaccinated (Matthijnssens et al., 2012). Similarly to Belgian data, some other research groups have also reported an apparently higher prevalence of G2P[4] in a population with high Rotarix® vaccine coverage (Carvalho-Costa et al., 2009; Kirkwood et al., 2011; Paulke-Korinek et al., 2013). In countries or regions with high RotaTeq® vaccine coverage, a higher prevalence of G3P[8] was reported. This phenomenon was observed in Australia and USA but there is as yet no clear explanation of this shift (Hull et al., 2011; Kirkwood et al., 2011; Matthijnssens et al., 2012). Although a slight increase/emergence of G3P[8] was noted in Slovenia in 2012, it is not likely that this was due to the use of RotaTeq® vaccine, since the coverage is too low to support such a conclusion. The vaccination coverage data for Slovenia includes both vaccines, suggesting that RotaTeq® vaccinated children are only part of it, minimizing the possible effect as it was observed in the regions with high vaccination coverage exclusively with RotaTeq®.

Despite the low vaccination coverage in Slovenia, it is important to follow the molecular epidemiology of rotaviruses. Vaccination coverage has been just below a quarter of eligible children in the last three years and a slight trend of decrease is observed. Since vaccination is on a voluntary basis, not covered by health insurance, this percentage can be expected to remain at or below the current level. Vaccine coverage in Slovenia is too low to confirm indisputably vaccine derived changes in the incidence or prevalence of rotavirus genotypes but some changes can be interpreted on the basis of country-specific data from countries with high vaccination coverage. Changes that were observed after vaccine introduction might thus be partially connected to selective vaccine pressure. The first change observed in the post-vaccination era was a fluctuation of rotavirus genotypes. The steep drop of G1P[8] prevalence from 74.1% in 2007/08 to 8.7% in 2010/11 may have been connected to vaccine introduction (Fig. 1A). Phylogenetic analysis of VP7 and VP8* genes supports this hypothesis, since rotavirus strains from the period before vaccination were grouped into the same lineage as the Rotarix® vaccine strain in both the VP7 gene and VP8* gene fragment (»Rotarix lineage«) (Figs. 2A and 4A). Although slight differences compared to the Rotarix® vaccine strain were observed, the antigenic epitopes of rotavirus strains before vaccination were almost identical to the Rotarix® vaccine strain. Only one amino acid difference was observed within the antigenic epitopes of Slovenian pre-vaccination G1P[8] strains compared to the Rotarix® vaccine strain in both VP7 and VP8* regions (Fig. 3). Starting from the 2007/08 season, the »Rotarix lineages combination« (VP7 G1 lineage II and P[8] VP8* lineage I) was detected in a minority of the G1P[8] strains analyzed (only three out of 25). It is difficult to understand whether such a shift in G1P[8] lineages is linked to the introduction of the vaccine, even at low or very low coverage, however similar patterns of lineage substitutions were observed in Belgium and Finland. In Belgium, Rotarix® is the most used vaccine, with high vaccine coverage, 88.5%. After the introduction of the Rotarix® vaccine, a shift was observed in the G1P[8] lineage, an increasing occurrence of lineage III being less related to the vaccine strain (Zeller et al., 2012). Similarly, Rotarix® was used in Finland from 2006 to 2008, with vaccine coverage of up to 29%, very similar to the Slovenian data. After the introduction of the Rotarix® vaccine, the majority of G1P[8] strains were not in lineage I, more related

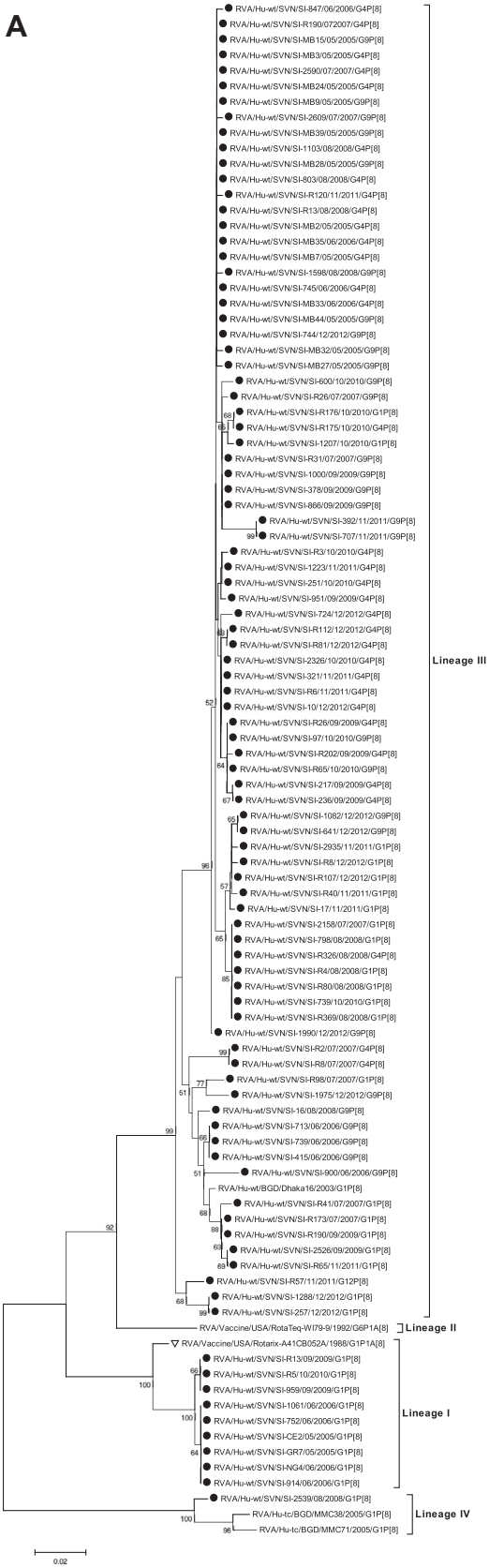

Fig. 4. Phylogenetic analysis of the VP4 (VP8* fragment) nucleotide sequence of (A) genotype G1P[8], G4P[8], G9P[8] Slovenian rotavirus strains and (B) genotype G2P[4] Slovenian strains with representative strains obtained from GenBank. Neighbor-joining phylogenetic trees were constructed with the Mega 5.2 software package, using the Kimura-2 parameter method with bootstrapping of 1000 replicates. SVN – a unique 3-letter country code for Slovenia.

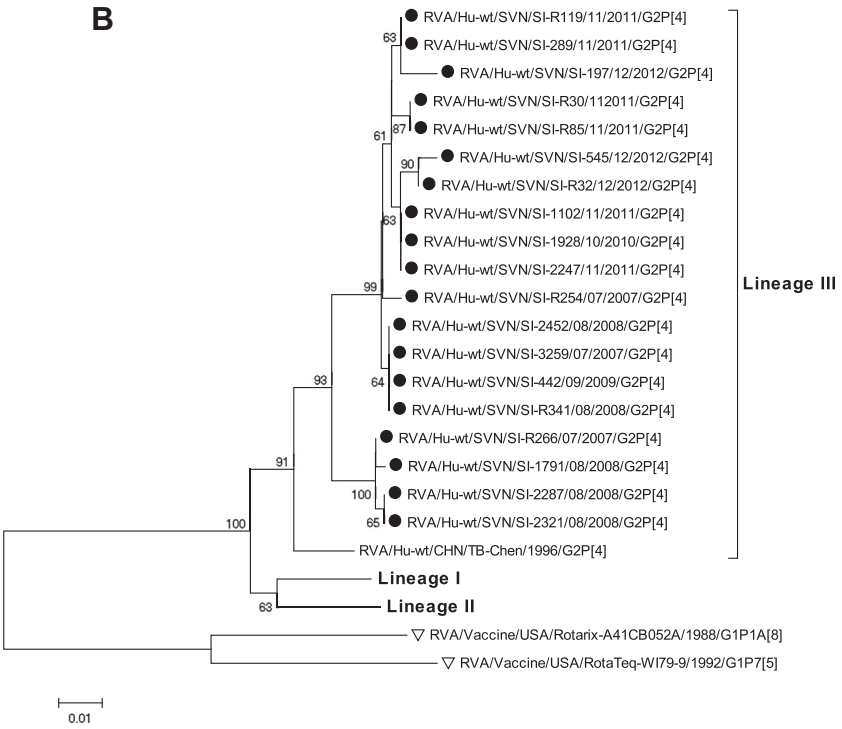

Fig. 4 (*continued*)

		8-1											8-2		8-3										8-4		
		100	146	148	150	188	190	192	193	194	195	196	180	183	113	114	115	116	125	131	132	133	135	87	88	89
	Rotarix_G1P[8]	D	S	S	N	S	S	A	N	L	N	N	E	R	N	P	V	D	S	S	N	D	N	N	T	N
	RotaTeq_P[8]	.	.	.	.	.	N	.	.	.	.	D	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G1	SI-R13/09	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
G1	SI-1061/06	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
G1	SI-R369/08	.	G	.	.	.	N	.	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G4	SI-R326/08	.	G	.	.	.	N	.	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G4	SI-R175/10	.	G	.	.	.	N	.	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G4	SI-R120/11	.	G	.	.	.	N	.	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G1	SI-R8/12	.	G	.	.	.	N	.	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G4	SI-R3/10	.	G	.	.	.	N	.	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G4	SI-MB39/05	.	G	.	.	.	N	.	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G9	SI-600/10	.	G	.	.	.	N	.	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G9	SI-392/11	.	G	.	.	.	N	.	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G9	SI-R65/10	.	G	.	.	.	N	T	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G4	SI-R202/09	.	G	.	.	.	N	T	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G4	SI-951/09	.	G	.	.	.	N	T	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G9	SI-97/10	.	G	.	.	.	N	T	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G4	SI-R26/09	.	G	.	.	.	N	T	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G4	SI-724/12	.	G	.	.	.	N	T	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G4	SI-236/09	.	G	G	.	.	N	T	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G1	SI-2935/11	.	G	.	.	.	N	.	.	.	D	G	.	.	.	.	.	.	N	G	.	.	D	.	.	.
G9	SI-MB32/05	.	.	.	.	.	N	.	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G9	SI-1598/08	.	G	.	.	.	N	.	D	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G1	SI-1288/12	.	G	.	.	.	N	.	.	.	.	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.
G1	SI-R173/07	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.
G4	SI-R8/07	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.
G9	SI-713/06	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.
G9	SI-16/08	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.
G9	SI-900/06	.	G	.	S	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.
G1	SI-R41/07	.	G	.	.	.	N	T	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.
G1	SI-2539/08	.	G	N	S	G	N	S	D	.	T	S	.	G	D	.	.	.	.	R	.	.	.	.	.	.
G2 P[4]	SI-R341/08	.	.	.	S	.	N	.	D	.	.	.	.	.	S	Q	T	N	N	E	.	S	D	.	.	D
G2 P[4]	SI-R254/07	.	G	.	S	.	N	.	D	.	.	.	.	.	S	Q	T	N	N	E	.	S	D	.	.	D
G2 P[4]	SI-2247/11	.	G	.	S	.	N	.	D	.	.	.	.	.	S	Q	T	N	N	E	.	S	D	.	.	D
G2 P[4]	SI-2321/08	.	G	.	S	.	N	.	D	.	.	.	.	.	I	Q	T	N	N	E	.	S	D	.	.	D

Fig. 5. Deduced amino acid sequence in the antigenic epitopes of the VP8* fragment of the VP4 protein.

to the Rotarix strain, but in lineage P[8]-III (Hemming and Vesikari, 2013). A change in VP8* sublineage has been temporally correlated to the use of Rotarix® in Finland (Hemming and Vesikari, 2013).

It is difficult to discriminate whether the increase of G2P[4] in the 2010/11 season was due to a natural fluctuation of genotype prevalence or to the effect of vaccination. However, in the neighboring country, Austria, where the Rotarix® vaccine is mainly used with a vaccine coverage of up to 84%, though the general prevalence of rotavirus genotypes is not known, G2P[4] genotype was mainly recorded in vaccination breakthrough (Paulke-Korinek et al., 2013). The authors reported that according to the EuroRotaNet database, the general prevalence of the G2P[4] genotype in Austria rose to as high as 73% after high vaccination coverage was reached (Paulke-Korinek et al., 2013).

No differences for genotype G4 and G9 strains were observed throughout the study period in terms of VP7 and VP8* molecular characteristics. Rotavirus G4 genotype strains were very homologous within the same sub-lineage Ib (Fig. 2C). It is not yet possible to draw conclusions from our data about the variation in G4 genotype prevalence during the study period. According to the phylogenetic analysis and antigenic epitopes, no specific clustering of G4 strains based on the year of detection was observed, in either VP7 or VP4 (VP8* fragment) genome segments. However, in a study of G4 strains from archived samples in a district area of Washington DC, co-circulation of different genetic variants within the defined time-frame was noted (McDonald et al., 2011). Whole genome analysis was performed in this study, elucidating genetic variability with possible genome re-assortment. The G4 genotype breakthrough in Slovenia appears to have happened for reasons other than variability within the VP7 and VP8*. Detailed whole genome analysis could be performed in the future to better characterize G4 strains circulating in the pre- and post-vaccination period. However, to the best of our knowledge, no study from countries or areas with high vaccination coverage have reported an increased prevalence of G4P[8] in unvaccinated or vaccinated children. No hint of vaccine derived changes in VP7 or VP8* were observed in G9 rotavirus strains either. In VP7, all G9 strains were found within lineage III, forming two clusters. Most of the strains were grouped in the SVN-major cluster. No specific pattern was observed in terms of the year of isolation or antigenic epitope differences between the two clusters, suggesting that genetic variances were located outside the antigenic epitopes. Since the same clustering of rotavirus G9 genotypes was also observed in the VP8* analysis, it is obvious that two variants of G9 rotavirus strains were circulating in Slovenia, with minor genetic changes during the study period.

Our data do not prove directly the influence of rotavirus vaccine on rotavirus strain diversity but provide additional post-marketing data for rotavirus vaccines in a country with low vaccination coverage. The influence of rotavirus vaccine in a similar, low vaccine coverage community was already shown in a study in Greece (Trimis et al., 2011). The vaccine coverage was reported to be lower than 30% but, according to hospital based data, a significant reduction of rotavirus gastroenteritis cases and the incidence of rotavirus gastroenteritis was observed after introduction of the vaccine. However, only minor changes in rotavirus genotype prevalence were observed, probably not connected to vaccine introduction. Although rotavirus vaccination coverage in Slovenia is low, presented data on rotavirus incidence and rotavirus hospitalization incidence among children 0–6 years appear to decline in the last few seasons (Fig. 1B). In the post-vaccine period, including the four rotavirus seasons 2009/10 to 2012/13 both calculated incidences remained at lower level than observed in the pre-vaccination period, despite of the genotype variability. This indicates that rotavirus vaccine in Slovenia might have an effect on the reduction of rotavirus disease burden. According to the data from our study, it is also highly possible that a selective influence of vaccination resulted in a changed molecular profile of circulating G1P[8] rotavirus strains.

Continuous monitoring of rotavirus genotypes is needed in the future to confirm the impact of even a low vaccination rate on rotavirus strain diversity. In addition, detailed molecular analysis of VP7 and VP4 genome segments should be performed regularly to track strain diversity and changed patterns of strains with altered antigenic epitopes. All these studies of antigenic epitope alterations based on deduced amino acid sequences should be further evaluated with cross-neutralization studies in order to gain real information on possible escape mutants in the future.

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