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Whole genome sequencing reveals genetic heterogeneity of G3P[8] rotaviruses circulating in Italy

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ABSTRACT

After a sporadic detection in 1990s, G3P[8] rotaviruses emerged as a predominant genotype during recent years in many areas worldwide, including parts of Italy. The present study describes the molecular epidemiology and evolution of G3P[8] rotaviruses detected in Italian children with gastroenteritis during two survey periods (2004–2005 and 2008–2013). Whole genome of selected G3P[8] strains was determined and antigenic differences between these strains and rotavirus vaccine strains were analyzed. Among 819 (271 in 2004–2005 and 548 in 2008–2013) rotaviruses genotyped during the survey periods, the number of G3P[8] rotavirus markedly varied over the years (0/83 in 2004, 30/188 in 2005 and 0/96 in 2008, 6/88 in 2009, 4/97 in 2010, 0/83 in 2011, 9/82 in 2012, 56/102 cases in 2013). The genotypes of the 11 gene segments of 15 selected strains were assigned to G3-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1; thus all strains belonged to the Wa genogroup. Phylogenetic analysis of the Italian G3P[8] strains showed a peculiar picture of segregation with a 2012 lineage for VP1-VP3, NSP1, NSP2, NSP4 and NSP5 genes and a 2013 lineage for VP6, NSP1 and NSP3 genes, with a 1.3–20.2% nucleotide difference from the oldest Italian G3P[8] strains. The genetic variability of the Italian G3P[8] observed in comparison with sequences of rotaviruses available in GenBank suggested a process of selection acting on a global scale, rather than the emergence of local strains, as several lineages were already circulating globally. Compared with the vaccine strains, the Italian G3P[8] rotaviruses segregated in different lineages (5–5.3% and 7.2–11.4% nucleotide differences in the VP7 and VP4, respectively) with some mismatches in the putative neutralizing epitopes of VP7 and VP4 antigens. The accumulation of point mutations and amino acid differences between vaccine strains and currently circulating rotaviruses might generate, over the years, vaccine-resistant variants.

1. Introduction

Group A rotavirus (RVA), family *Reoviridae*, is the major etiological agent of acute gastroenteritis in children during the early years of life (Estes and Greenberg, 2013).

The RVA genome is surrounded by a triple layered capsid, and is made up of 11 segments of double-stranded RNA (dsRNA), which encode six structural viral proteins (VP1–VP4, VP6 and VP7) and five or six non-structural proteins (NSP1–NSP5 and sometimes NSP6) (Estes and Greenberg, 2013).

The traditional binary classification system for RVAs is based on the two outer capsid proteins VP7 (glycoprotein, G-genotype) and VP4 (protease sensitive protein, P-genotype). While at least 73 G/P genotype combinations have been described in humans (Matthijnssens et al., 2011), only a limited number of strains – G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and to a lesser extent G12P[8] and G12P[6] – are most frequently associated with the RVA disease burden globally (Gentsch et al., 2005; Santos and Hoshino, 2005; Patel et al., 2011; Bányai et al., 2012; Cilla et al., 2013). A more complete RVA classification assigns genotypes to all 11 dsRNA segments, providing an opportunity to better characterize RVA strains and describe the molecular epidemiology of RVA infections in a greater detail (Matthijnssens

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et al., 2008). Based on complete RVA genome sequence comparison, three human genotype constellations of the non-G, non-P genes have been described: Wa-like (genotype 1), DS-1-like (genotype 2), and AU-1-like (genotype 3).

G3 genotype presents the broadest host range and, consequently, the highest number of P type associations described for any other RV G type (Martella et al., 2003, 2010; Lee et al., 2003; Gentsch et al., 2005). Despite this high diversity, the majority of human G3 strains carries the P[8] VP4 genotype. G3P[8] strains have been found to circulate at low rates during the 1990s globally, except some Far East Asian countries. The re-emergence of G3P[8] RVAs in humans has been widely documented in the last decade (Ngo et al., 2009; Hull et al., 2011; Kirkwood et al., 2011; Mitui et al., 2011; Stupka et al., 2012; Gómez et al., 2014). The increased frequency of this strain in most of the reporting countries is supposed to be at expenses of the decrease of frequencies of G1P[8] and G9P[8] strains (Mitui et al., 2011; Stupka et al., 2012).

Two live-attenuated RVA vaccines, Rotarix (G1P[8]) and RotaTeq (G1-G4, P[8]), have been successfully introduced in many countries worldwide, including Italy. The parental RVA strains used to generate the vaccines were isolated more than 20 years ago and, at present, little is known about the relationship between currently circulating human RVAs and the vaccine strains.

The present study describes the molecular epidemiology of G3P[8] RVAs detected in Italian children with gastroenteritis during two survey periods (2004–2005 and 2008–2013). The whole genome of representative G3P[8] RVAs was determined. Also, the antigenic/genetic differences between G3P[8] RVAs circulating in Italy and vaccine RVA strains were investigated.

2. Material and methods

A total of 5729 children (range: 1 month–13 years and 6 months, median age: 4 years and 2 months) with gastroenteritis, as inpatients or outpatients at the Parma University Hospital, Northern Italy, was screened during 2004–2013 for RVA in stools by electron microscopy and latex agglutination (Orion Diagnostica). Molecular data were generated for the RVAs detected during 2004–2005 and 2008–2013. The stools resulted RVA positive in 2006 and 2007 were not available for genetic analyses. For RVA genotyping, viral RNA was extracted from stools using the RNaid Kit (BIO101), according to the manufacturer's instructions, and submitted to G/P typing protocols by multiplex semi-nested reverse transcription-PCR strategy (Medici et al., 2007; Ruggeri et al., 2011). Laboratory diagnosis for RVA infection was performed upon specific medical request. Patients' identity and subsequent medical information was adequately protected and remained anonymous during the study.

For full-genome sequencing of G3P[8] RVAs, reverse transcription was performed using AMV reverse transcriptase (Promega) with random hexamer tailed by a common PCR primer sequence (Djikeng et al., 2008). Polymerase chain reaction was carried out with Taq DNA polymerase (Thermo Scientific) applying the following thermal profile: initial denaturation (95 °C for 3 min) was followed by 40 cycles of amplification (95 °C for 30 s, 48 °C for 30 s, 72 °C for 2 min) and terminated at 72 °C for 8 min.

Approximately 30 to 40 ng cDNA obtained by the random RT-PCR were subjected to enzymatic fragmentation and adaptor ligation using the reagents supplied in the NEBNext Fast DNA Fragmentation & Library Prep Set for Ion Torrent kit (New England Biolabs) according to the manufacturer's instruction. Barcoded adaptors were retrieved from the Ion Xpress™ Barcode Adapters kit (Life Technologies). After size selection emulsion PCR was carried out on a OneTouch v2 instrument according to the manufacturer's protocol (Life Technologies). Templated beads were enriched on an Ion One Touch ES machine. The sequencing protocol recommended for the Ion PGM™ Sequencing 200 Kit v2 on a 316 v2 chip was strictly followed. Raw sequence data were mapped onto reference G3P[8] RVA sequences obtained from the GenBank in the CLC Genomics Workbench version 7 (http://www.clcbio.com/). After visual inspection of the short-read sequence alignments, a single consensus sequence was finalized for each strain. The GenBank accession numbers of the G3P[8] Italian strains are KT988141–KT988305.

Multiple sequence alignments and phylogenetic tree constructions were performed with MEGA software (6.06) (Tamura et al., 2013), applying the maximum-likelihood method. Reliability of the phylogenetic trees was assessed by bootstrap re-sampling over 1000 replicates.

3. Results and discussion

After the introduction of RVA vaccines, surveillance for RVA has been intensified worldwide in order to gain pivotal information on the impact of vaccines on the epidemiology of RVA strains in the post-vaccine era (Bányai et al., 2012; Dóró et al., 2014).

In the prefecture of Parma (Emilia Romagna), Northern Italy, RVA vaccines were introduced officially in November 2006, with the approval of local health authorities. Although the RVA vaccines are strongly recommended by family pediatricians, they are not mandatory and therefore the vaccination coverage is not high. National and local data about vaccination coverage and data on the RVA prevalence after vaccine introduction are not available. A marked geographic heterogeneity in immunization practices has emerged in Italy as a consequence of the decentralization of the national health system. Only five (24%) regions (Lazio, Toscana, Basilicata, Piemonte, and Puglia) have included RVA vaccinations in their routine immunization schedules (Alfonsi et al., 2011). In our survey, the percentage of RVA infections in children with gastroenteritis showed a nearly three-fold reduction within a few years after the introduction/recommendation of RVA vaccines. In the pre-vaccine era the highest percentage of 36.7% was observed in 2006, while it decreased to 13.5% in 2013 (Table 1).

Among 819 genotyped RVA strains (271 in 2004–2005 and 548 in 2008–2013), significant annual changes of the G3P[8] relative proportion were observed. No G3P[8] RVA was detected in the years 2004, 2008 and 2011, whilst the G3P[8] detection rate was 16% in 2005, 6.8% in 2009, 4.2% in 2010, 11% in 2012 and 54.9% in 2013. Interestingly, G3P[8] became the predominant genotype during 2013, displacing the previous dominant G1P[8] genotype. The G1P[8] detection rate remained high even after the introduction of vaccines and decreased sharply from 96.4% in 2011 to 6.9% in 2013. Although, a limitation of the present study was the lack of a continue genotyping survey (no molecular data were generated from 2006 to 2007) the portrayed epidemiological picture can be considered representative of the RVAs circulating in the last decade in Parma. Our findings seem to reflect the trends described globally, confirming the increasing G3P[8] prevalence in the recent years (Hull et al., 2011; Kirkwood et al., 2011; Mitui et al., 2011; Stupka et al., 2012; Gómez et al., 2014). The increase of the number of G3P[8] cases in 2013 was not associated with a significant increase of the frequency of RVA detection. Likewise, changes in the rates of detection of G1P[8] cases over the years did not correlate with changes in RVA prevalence.

The percentage of RVA infections in children with gastroenteritis significantly decreased after the introduction of RVA vaccines, remaining under 16.4% after 2008. Continual monitoring is needed to understand the patterns and trends of the circulating RVA genotypes, chiefly for common G1P[8] and G3P[8] strains that showed significant yearly fluctuation in their prevalence.

Out of the 105 G3P[8] RVAs, 15 G3P[8] strains (at least two strains per year: two G3P[8] of 2005, four of 2009, two of 2010, two of 2012 and five of 2013) were randomly selected for full-genome sequencing. Upon genome sequence and phylogenetic analyses, all the 15 G3P[8] RVA strains were assigned to the gene constellation I1-R1-C1-M1-A1-

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Table 1
Distribution of rotavirus G and P types detected in Italian children with gastroenteritis in 2004–2005 and 2008–2013.

G/P type	Year	2004	2005	2008	2009	2010	2011	2012	2013	Total
	No. of RVA detected (%)	83 (27.1)	188 (34.1)	96 (16.4)	88 (16)	97 (16)	83 (14)	82 (11.6)	102 (13.5)	819 (17.6)
	No. of children with gastroenteritis	306	551	586	551	606	597	705	758	4660
G1P[4]								2 (2.4)	7 (6.9)	9 (1.1)
G1P[8]		56 (67.5)	60 (31.9)	65 (67.7)	37 (42)	69 (71.1)	80 (96.4)	42 (51.2)	7 (6.9)	416 (50.8)
G2P[4]		3 (3.6)	5 (2.7)	1 (1)	10 (11.4)	1 (1)	1 (1.2)	5 (6.1)	7 (6.9)	33 (4)
G3P[4]						1 (1)				1 (0.1)
G3P[6]						1 (1)				1 (0.1)
G3P[8]			30 (16)		6 (6.8)	4 (4.2)		9 (11)	56 (54.9)	105 (12.8)
G4P[8]		13 (15.7)	2 (1)	2 (2)	9 (10.3)	19 (19.6)		9 (11)	18 (17.6)	72 (8.8)
G6P[9]								1 (1.2)		1 (0.1)
G8P[14]		1 (1.2)			1 (1.1)					2 (0.4)
G9P[8]		3 (3.6)	78 (41.5)	6 (6.3)	5 (5.8)				2 (2)	94 (11.4)
G10P[14]					1 (1.1)					1 (0.1)
G12P[6]									2 (2)	2 (0.4)
G12P[8]									2 (2)	2 (0.4)
Mixed G/P		7 (8.4)	13 (6.9)	22 (23)	19 (21.5)	2 (2.1)	2 (2.4)	14 (17.1)	1 (0.9)	80 (9.7)

RVA: rotavirus group A. RVA prevalence in 2006: 36.7% (211/575). RVA prevalence in 2007: 22.1%.(109/494).

N1-T1-E1-H1. This finding indicated that the G3P[8] RVAs belong to genotype 1 (Wa-like).

Phylogenetic analysis showed that the 15 G3P[8] Italian strains clustered within G3-Ia lineage (99.1–100% nucleotide identity) in the VP7 tree and all the strains were grouped into the P[8]-III lineage (97.1–100% nucleotide identity) in the VP4 tree, except a 2009 strain, which segregated in the P[8]-IV lineage (Fig. 1).

When analyzing the backbone genes, the strains detected in 2005 and 2009–2010 clustered in a single common lineage and were more closely related to contemporary G3P[8] detected globally. On the other hand, the Italian G3P[8] strains detected in 2012–2013 showed a peculiar pattern of segregation, with point mutations involving different gene segments. Thus, the VP1, VP2, VP3, NSP1, NSP2, NSP4 and NSP5 genes of the G3P[8] strains from 2012 segregated in a lineage divergent from the oldest Italian strains (1.3–20.2% nucleotide difference). Likewise the 2013 G3P[8] RVAs grouped into a separate lineage from the remaining Italian G3P[8] RVAs when analyzing the VP6, NSP1, and NSP3 genes (4.6–18% nucleotide difference). Interestingly, the additional lineages of 2012 and 2013 RVAs were closely related in some genes (VP1, VP3, VP6, and/or NSP4) to the ancestral Wa strain detected in 1974. This clearly suggests that the genome configuration of G3P[8] RVA has evolved over the years due to mechanisms of genetic drift intermingled with intra-typic reassortment, although, owing to the limited genome sequence data still available in GenBank, it is impossible to determine the exact temporal order of these events.

In the present study, for most gene segments different lineages were found in the Italian G3P[8] strains, as observed in other large-scale genomic studies investigating Wa-like RVAs (McDonald et al., 2009; Roy et al., 2014; Zhang et al., 2014; da Silva et al., 2015; Zeller et al., 2015). The genetic variability of the Italian G3P[8] observed in comparison with sequences of rotaviruses available in GenBank suggested a process of selection acting on a global scale, rather than the emergence of local strains, as several lineages were already circulating globally.

Finally, all the 15 Italian G3P[8] strains clustered in a branch different from those of the VP7 gene of the pentavalent RotaTeq (5–5.3% nucleotide difference and 2.8–3.4% amino acid difference) and in a lineage different from those of the VP4 gene of the RotaTeq and Rotarix vaccines (7.2–11.4% nucleotide difference and 6.8–9.7% amino acid difference). Intra-genotypic differences (3 aa changes in VP7 and 4–9 aa changes in VP4) were found within the putative neutralizing epitopes of both antigens between the Italian G3P[8] strains and the RVA vaccines (Figs. 2 and 3). Structural analyses are required to assess if amino acid sequence changes in antigenic epitopes resulted in antigenic differences between the vaccine and G3P[8] RVA strains currently circulating in Italy.

Lineage replacement has been suggested as an important evolutionary mechanism for RVAs to adapt to different immunological environments and the observed changes in RVA strains and lineages could be the result of natural fluctuations or the effect of vaccine-driven evolution (McDonald et al., 2009; Dennis et al., 2014; Zhang et al., 2014; Magagula et al., 2015; Zeller et al., 2015). Whether protective immunity elicited by RVA vaccines may be affected not only by differences in the VP7/VP4 types but also by different intra-genotypic lineages is unclear. In this study we did not observed changes in the VP7 and VP4 putative epitopes between the G3P[8] RVAs circulating before and after vaccine introduction, even if they differ in VP7 and VP4 antigens from vaccine strains. Whether, over time, these differences may result in selection of strains able to escape the RVA neutralizing-antibody pressure induced by vaccines remains to be investigated (Zeller et al., 2012).

4. Conclusions

This study indicates that G3P[8] RVA strains tend to maintain a stable Wa-like constellation, although the emergence of novel lineages, evolving through accumulation of point mutations and reassortment with older Wa-like RVAs, can affect in different ways virus–host relationship. Genetic heterogeneity was observed in G3P[8] RVAs in the VP6 gene, encoding the major capsid protein, in 2013, but not in 2012. This pattern was unique for the 2013 G3P[8] RVAs and it was the only hallmark we could associate with the spread of G3P[8] strains in 2013. This would imply mechanisms that influence the efficacy of virus neutralization mediated by VP6-specific antibodies (Burns et al., 1996), and confirm that besides the surface proteins VP7 and VP4, also other viral proteins could be involved in immune defense (Ward et al., 1993; Franco et al., 1997; Bernstein and Ward, 2004; Corthésy et al., 2006). Further investigation of all relevant antigens is needed for a better understanding of the immune defense against RVAs.

Whole genome characterization of RVA strains nicely complements routine VP7 and VP4 genotyping assays and is pivotal to track the emergence of novel RVA strains and to assess the long-term evolution of common RVA strains.

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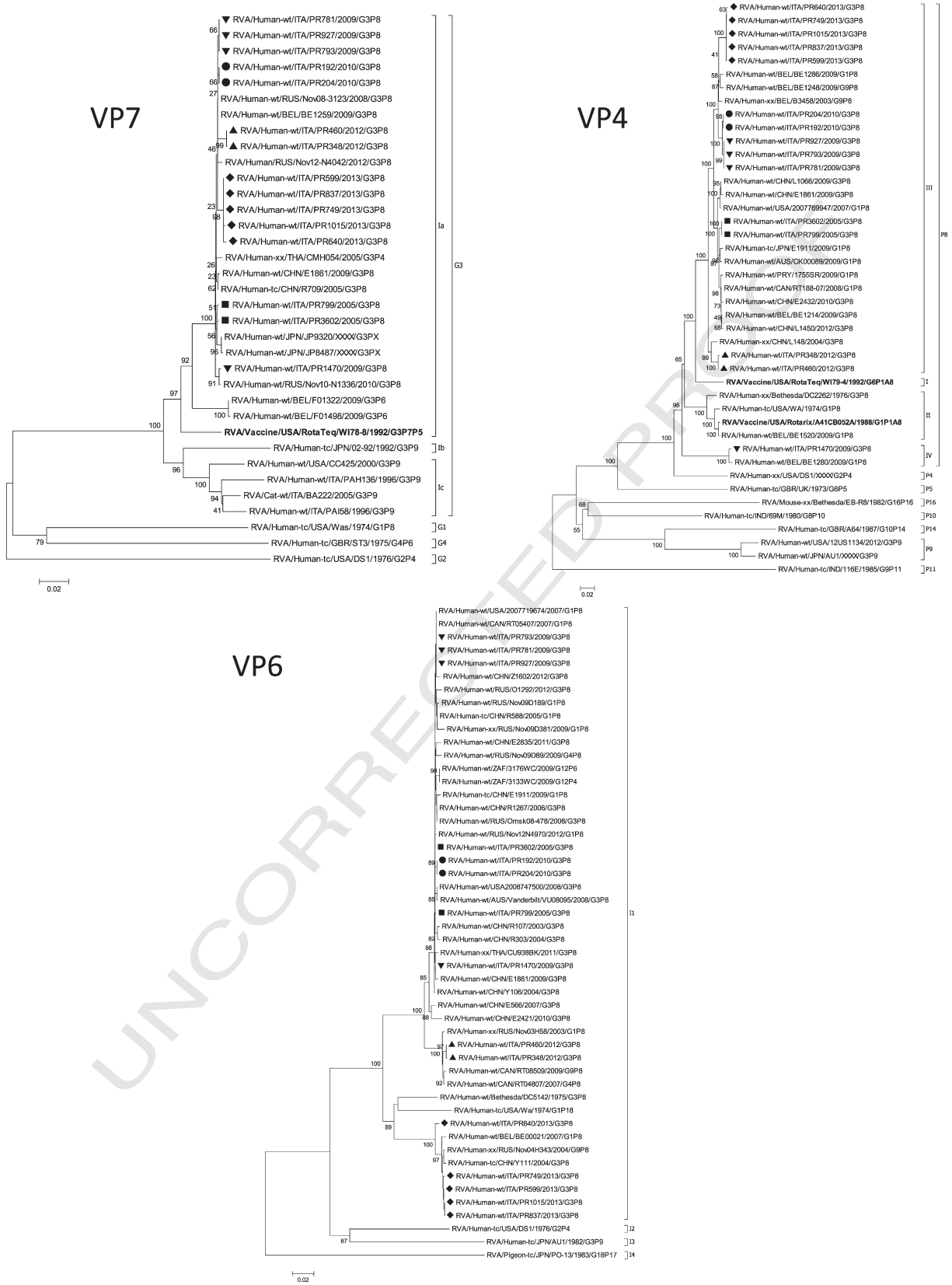

Fig. 1. Phylogenetic dendrograms based on the nucleotide sequences of all 11 gene segments of rotaviruses. Italian G3P[8] rotaviruses detected in 2005 (■), in 2009 (▼), in 2010 (●), in 2012 (▲) and in 2013 (♦). In VP7 and VP4 vaccines strains are reported in bold. Trees were built with the maximum-likelihood method, and bootstrapped with 1000 repetitions. Bootstrap values are indicated at nodes of branches. Bar, number of nucleotide substitutions per site.

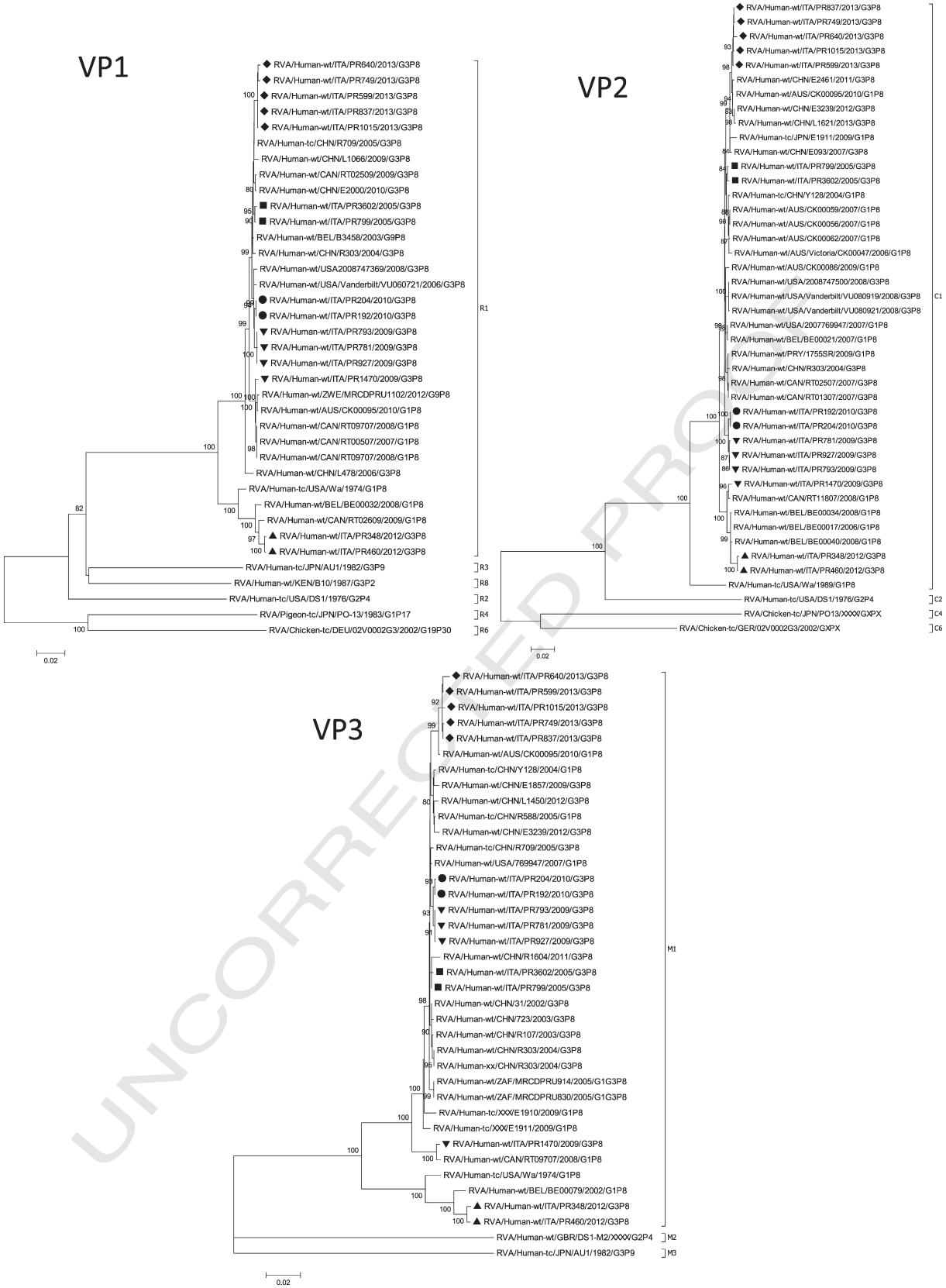

Fig. 1 (*continued*).

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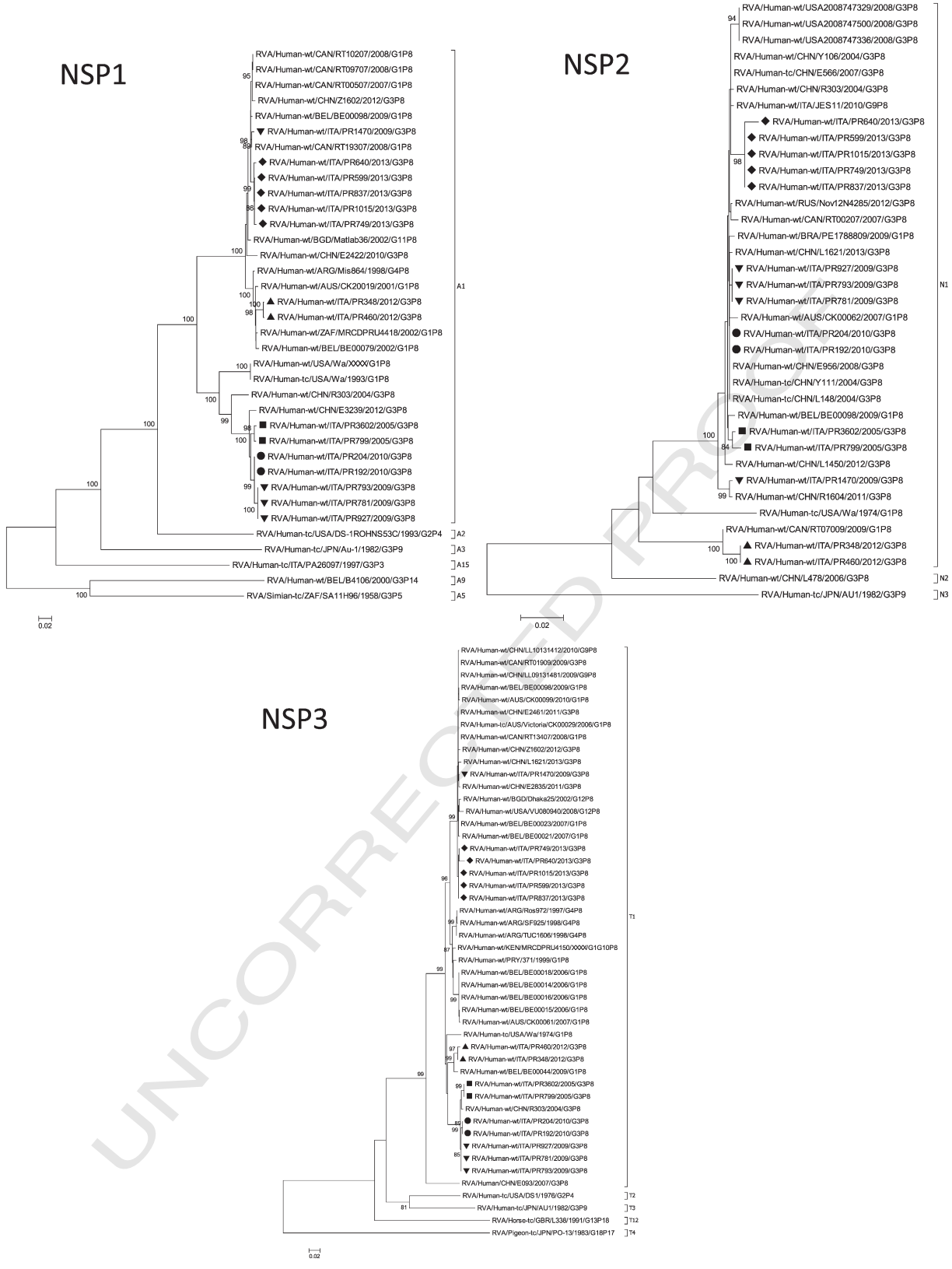

Fig. 1 (*continued*).

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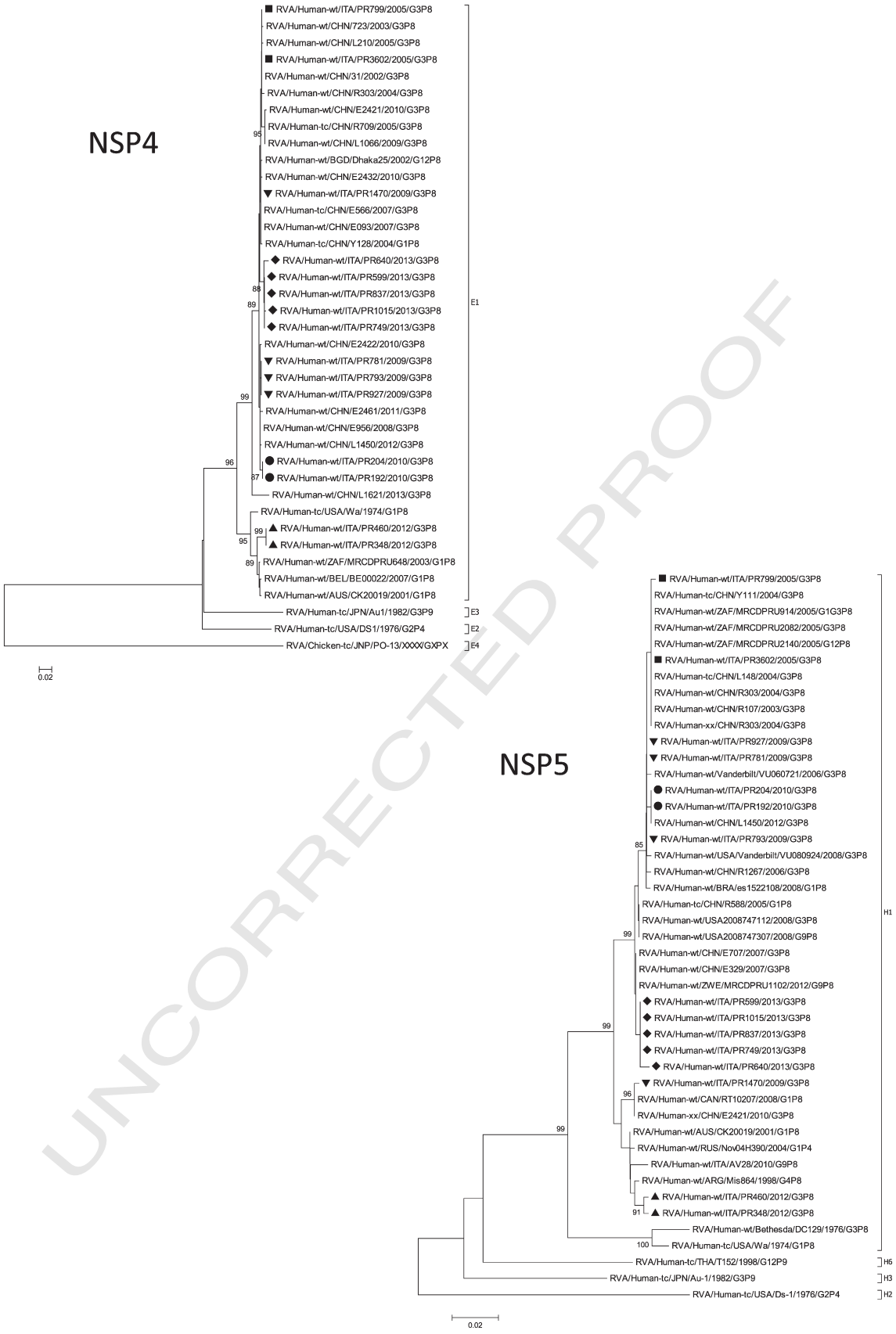

Fig. 1 (*continued*).

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	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
RotaTeq-WI78-9/G3P[5]	T	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	A	N	K	D	K	D	A	T	L	S	E	A	G
PR799/2005	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.
PR3602/2005	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.
PR793/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.
PR781/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.
PR1470/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.
PR927/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.
PR204/2010	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.
PR192/2010	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.
PR348/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.
PR460/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.
PR640/2013	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.
PR749/2013	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.
PR837/2013	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.
PR599/2013	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.
PR1015/2013	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.

Fig. 2. Alignment of the amino acid residues defining the neutralization domains (designated as 7-1a, 7-1b and 7-2) in VP7 of 13 Italian G3P[8] rotaviruses with those of the G3 strain of RotaTeq™ vaccine. Dots indicate conserved amino acids.

	VP8*																									VP5*																	
	8-1											8-2		8-3											8-4			5-1												5-2		5-3		5-4		5-5	
	100	145	147	149	187	189	191	192	193	194	195	179	182	113	114	115	116	125	131	132	133	135	87	88	89	383	385	387	392	393	397	439	440	433		458		428		305	
Rotarix-A4JCB052A/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	S	Y	S	A	W	N	L	R	E		N		S		L	
RotaTeq-W179-4/G6P[8]	D	S	S	N	S	N	A	N	L	N	D	E	R	N	P	V	D	N	R	N	D	D	N	T	N	R	H	S	A	W	N	L	R	E		N		S		L	
PR799/2005	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.		.		.		.	
PR3602/2005	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.		.		.		.	
PR793/2009	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.		.		.		.	
PR781/2009	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.		.		.		.	
PR1470/2009	.	G	N	S	G	N	S	D	.	T	S	.	G	D	.	.	.	S	R	.	.	N	.	.	.	.	D	T	.	.	.	.	.	.		.		.		.	
PR927/2009	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.		.		.		.	
PR204/2010	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.		.		.		.	
PR192/2010	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.		.		.		.	
PR348/2012	.	G	.	.	.	N	.	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.		.		.		.	
PR460/2012	.	G	.	.	.	N	.	.	.	D	G	.	.	.	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.		.		.		.	
PR640/2013	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.		.		.		.	
PR749/2013	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.		.		.		.	
PR837/2013	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.		.		.		.	
PR599/2013	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.		.		.		.	
PR1015/2013	.	G	.	.	.	N	.	.	.	.	G	.	.	D	.	.	.	N	R	.	.	D	.	.	.	.	D	.	.	.	.	.	.	.		.		.		.	

Fig. 3. Alignment of the amino acid residues defining the neutralization domains in VP8* (designated as 8-1, 8-2, 8-3, 8-4) and VP5*(designated as 5-1, 5-2, 5-3, 5-4, 5-5) epitopes of VP4 of 13 Italian G3P[8] rotaviruses with those of P[8] strains of Rotarix™ and RotaTeq™ vaccines. Dots indicate conserved amino acids.

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