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Detection of Unusual G6 Rotavirus Strains in Italian Children With Diarrhoea During the 2011 Surveillance Season

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Two rare G6 rotavirus A (RVA) strains, designated as RVA/human-wt/ITA/CEC06/2011/G6P[6] and RVA/human-wt/ITA/PG05/2011/G6P[9], were identified in stool specimens from children hospitalized in Central Italy. After PCR genotyping, the samples CEC06 and PG05 gave G-UD-P[6] and G-UD-P[9] genotypes, respectively. To determine the G-type and to characterize further the two strains, sequencing of 8 of the 11 genomic segments was performed. CEC06 and PG05 strains were found to possess unusual genotype constellations: G6-P[6]-I2-A2-N2-T2-E2-H2 and G6-P[9]-I2-A3-N2-T3-E3-H3, respectively. This study reports the first detection of rare G6P[6] and G6P[9] RVA strains in peninsular Italy. Phylogenetic analysis of VP4 (VP8*), VP7, VP6, and NSP1-5 showed no evidence of zoonosis or interspecies reassortment, revealing for both strains constellations previously associated to human cases. ***J. Med. Virol. 85:1860–1869, 2013.***
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KEY WORDS: group A rotavirus; sequence; HBGA; G6P[6]; G6P[9]; Italy

INTRODUCTION

Group A rotaviruses (RVA) are the leading cause of acute gastroenteritis (GE) in young (<5 years of age) children, causing approximately 450 000 deaths worldwide, mostly in developing countries [Tate et al., 2012]. RVA virions consist of icosaedral, triple-layered, and non-enveloped particles possessing a segmented genome made of 11 double-stranded RNA linear segments [Estes and Cohen, 1989]. The RVA outer layer is composed of two proteins, VP7 and VP4, expressed by gene 9 and 4, respectively. VP7 (G-type) and VP4 (P-type) genotypes are the basis for the binary RVA nomenclature [Hoshino et al., 1985]. Although at least 27 G-types and 37 P-types of rotavirus have been reported [Matthijnssens et al., 2011; Trojnar et al., 2013], most of RVA infections in humans worldwide are related to five major G/P combinations: G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] [Gentsch et al., 2005]. However, besides strains common worldwide such as G1P[8], G3P[8], and G9P[8], human RVA genotypes circulating in the African countries also include strains which are unconventional in Europe and in other developed areas of the world, with a particularly high prevalence of G2P[6] and G8P[6] [Santos and Hoshino, 2005; Todd et al., 2010]. Facing the unusual and emerging RVA combinations that are increasingly reported in humans, a new nomenclature system has been adopted, based on nucleotide sequences and genotypes of each of the 11 genomic segments [Matthijnssens et al., 2008a]. This system facilitates

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investigation of the origin of new and potentially epidemic RVA genotypes, and the evolutionary mechanisms these undergo from interspecies transmission through adaptation to human hosts, including gene reassortment [Martella et al., 2010].

Despite the large number of genotypes established for both VP7 and VP4 genomic segments, comprehensive analysis of sequences for the entire rotavirus genome available in GenBank suggests that in the majority of cases rotaviruses can be assigned to two main constellations of genotypes, corresponding to previously known subgroup I and II of rotavirus prototype strains, Wa and DS-1 [Kalica et al., 1981]. The former exhibits the G1-P [8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 formulation, and the latter is reported as G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2 [Matthijnssens et al., 2011].

In addition, the unique rotavirus strain AU-1 found in Japan in 1985 [Matthijnssens and Van Ranst, 2012] is indicated as G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H3.

In 2006, two RVA vaccines were licensed for human use, and both are now used in an increasing number of countries worldwide. Their antigenic compositions are based on the commonest RVA genotypes circulating worldwide, the monovalent vaccine Rotarix® being based on human G1 and P[8] antigen specificities, whereas the bovine-derived pentavalent reassortant vaccine Rotateq® is based on G1, G2, G3, G4, and P[8] antigens [Ruiz-Palacios et al., 2006; Vesikari et al., 2006; Linhares et al., 2008]. The efficacy of both vaccines is very high against diarrhoea caused by the common rotavirus strains, including G9 RVA which has emerged and spread rapidly in the 2000s, and has now established as a major global genotype in humans [Santos and Hoshino, 2005; Gentsch et al., 2009; Matthijnssens et al., 2010; Iturriza-Gomara et al., 2011].

Recently, novel uncommon RVA genotypes such as G6, G8, and G12, in association with P[6], P[8], and P[14], have been reported in sporadic cases of GE in several countries, particularly in developing areas [Gerna et al., 1994; Iturriza Gomara et al., 2004; Matthijnssens et al., 2006; Matthijnssens et al., 2010; Nordgren et al., 2012b]. Due to their unconventional G- and P-genotypes, these are regarded as possible epidemic strains potentially able to escape the immune protection sustained by current vaccines [Offit and Dudzik, 1988; Ijaz et al., 1991; Dunn et al., 1995; Ward et al., 2006]. These strains are possibly generated through animal–human transmission involving reassortment events with multiple strain infections [Martella et al., 2010].

During the surveillance activity of RotaNet-Italy, two RVA strains with unusual G/P combinations G6P[6] and G6P[9] were found in two distinct cities of Central Italy in 2011 [Ruggeri et al., 2011]. To understand better their origin, the two strains were subjected to sequencing of the genes encoding for the main structural proteins VP7, VP4, and VP6, and for the non-structural proteins NSP1-5.

MATERIALS AND METHODS

Clinical Cases, and RVA Identification

Stool specimens were collected on admission from two children with severe disease, aged 10 months and 21 months, respectively, who were admitted with acute GE to public hospitals of Cecina (Tuscany) and Perugia (Umbria), in 2011. Clinical information was obtained from the anamnesis forms of the RotaNet-Italy surveillance project filled by the pediatric units, in compliance with the Informed Consensus Agreement. Additional information including patients' histo-blood group (routinely performed according to the hospital standard operative procedures for patients' management) was supplied by personal communications with the parents of both children.

Rotavirus infection was diagnosed at the hospital pediatric units by the commercial antigen detection methods in daily use.

Reverse Transcription-Polymerase Chain Reaction and Nucleotide Sequencing

Total viral RNA was extracted from 140 μl of 10% fecal suspensions in H_2O, using the Viral RNeasy Mini Kit (Qiagen, Milan, Italy), according to the manufacturers' instructions. RNA was eluted in 60 μl of RNase-free water, and stored at −80°C until use.

G- and P-genotyping was performed by reverse transcription nested polymerase chain reaction (RT-nPCR), using mixtures of primers for either gene 9 and 4, as described previously [Gentsch et al., 1992; Iturriza-Gomara et al., 2004]. RT-PCR was performed with the Access RT-PCR kit (Promega, Madison, WI) following the manufacturers' instructions.

For sequence analysis, RT-PCR reactions included primers specific for each of the eight genes investigated [Matthijnssens et al., 2008a], using a T_m of 50°C for all the reactions. A 3 min elongation step was used to obtain VP4 (VP8*), VP7, and NSP2-5 amplicons, whereas for VP6 and NSP1, elongation was protracted for 6 min. PCR products were visualized under UV light after electrophoretic separation on a 2% agarose gel stained with ethidium bromide.

Nucleotide sequencing of genes amplified was performed by Macrogen, Inc. (Seoul, South Korea), using the same primers used for PCR. The sequencing reaction was based on the BigDye chemistry, and all sequences were performed twice.

Software Analysis

The sequencing files obtained were analyzed and corrected with ChromaPro 2.23 (Technelysium, Queensland, Australia), and consensus sequences were obtained using SeqMan II (DNASTAR, Madison, WI). Multiple sequence alignments, phylogenetic analysis and analysis of the amino acids within the VP8* binding domain were performed using the MEGA5 software (www.megasoftwares.com).

Sequences obtained in this study are available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers:

CEC06_VP8 Acc. Num. KC152908; CEC06_VP7 Acc. Num. KC152909; CEC06_VP6 Acc. Num. KC152910; CEC06_NSP1 Acc. Num. KC152911; CEC06_NSP2 Acc. Num. KC152912; CEC06_NSP3 Acc. Num. KC152913; CEC06_NSP4 Acc. Num. KC152914; CEC06_NSP5 Acc. Num. KC152915.

PG05_VP8 Acc. Num. KC152916; PG05_VP7 Acc. Num. KC152917; PG05_VP6 Acc. Num. KC152918; PG05_NSP1 Acc. Num. KC152919; PG05_NSP2 Acc. Num. KC152920; PG05_NSP3 Acc. Num. KC152921; PG05_NSP4 Acc. Num. KC152922; PG05_NSP5 Acc. Num. KC152923.

PG01_VP8 KC152924; PG01_VP7 Acc. Num. KC152925; PG01_VP6 Acc. Num. KC152926; PG01_NSP1 Acc. Num. KC152927; PG01_NSP2 Acc. Num. KC152928; PG01_NSP3 Acc. Num. KC152929; PG01_NSP4 Acc. Num. KC152930; PG01_NSP5 Acc. Num. KC152931.

RESULTS

Two unconventional rotaviruses were detected in the stools of a child born in Cecina, Tuscany, to Senegalese parents (CEC06/2011), and of a child born in Perugia, Umbria, to Rumanian parents (PG05/2011). Both children were less than 1 year old, had not received rotavirus vaccination, and were admitted to public hospitals with a diagnosis of acute GE and dehydration. The child from Cecina presented acute osmotic shock, with severe risk for his life. The disease course was normal in both cases, with no complication after the oral rehydration therapy. Both children belonged to the A-type histo-blood group. Neither children had history of travel to their country of origin, nor did they have animals at home.

After the first cycle of PCR genotyping, the G/P combination found for the strains was G-UD-P[6] and G-UD-P[9], respectively.

In both cases, the VP7 genotype was determined following sequence analysis of the full gene 9 RT-PCR product, yielding G6P[6] for strain RVA/human-wt/ITA/CEC06/2011, and G6P[9] for strain RVA/human-wt/ITA/PG05/2011.

In addition to genomic segments 9 and 4, genome segments encoding VP6 and NSP1-5 were also amplified by RT-PCR, and subjected to nucleotide sequencing. Although a full-genome sequencing was not performed for the two Italian strains, analysis of 8 of their 11 genomic segments permitted to address possible reassortment involving large part of the viral genes. The genotyping and phylogenetic analysis of the eight genes investigated for strains RVA/human-wt/ITA/CEC06/2011 and RVA/human-wt/ITA/PG05/2011 revealed the presence of two distinct human RVA genomic constellations not detected previously in Italy: that is, G6-P[6]-I2-A2-N2-T2-E2-H2 and G6-P[9]-I2-A3-N2-T3-E3-H3 (Fig. 1a–h).

The VP7 tree (Fig. 1a) revealed that the two Italian G6 strains clustered strictly together in a cluster that included the human G6 strains reported during the last 10 years in Europe, Japan, and Africa, showing the highest nucleotide similarity among all genes investigated (96%). The VP4 (VP8*) tree (Fig. 1b) showed that the CEC06/2011 rotavirus belonged to the P[6] genotype, clustering strictly with P[6] strains circulating in Africa, Bangladesh, Belgium, and USA, whereas strain PG05/2011 clustered in the P[9] group, with a gene 4 closely similar to several human G3P[9] and G6P[9] strains, identified either in Japan or in the Italian island of Sicily, and to a feline G3P[9] strain reported in Southern Italy.

In addition to VP7, strains CEC06/2011 and PG05/2011 shared the same genotype only for VP6 and NSP2 (Fig. 1c and e), but they appeared to belong to two different clusters, presenting a low similarity rate (i.e., 92% and 90%).

Phylogenetic analysis of NSP1 and NSP3-5 (Fig. 1d, f–h) revealed that the two Italian strains clustered separately because of their different genotype: strain CEC06/2011 belonged to the genotype 2 for all genes, whereas strain PG05/2011 genes clustered consistently with the genotype 3 group.

In all trees, strain CEC06/2011 showed the highest similarity with the G6P[6] strains identified in Burkina Faso (BFA) in 2010 (similarities between 99% and 100%) [Nordgren et al., 2012b], whereas strain PG05/2011 correlated strictly with the 2010 Japanese strain KF17 [Yamamoto et al., 2011].

To address possible interspecies reassortments involving the Italian G6 strains, a typical DS-1 like strain circulating in Central Italy in 2011 (RVA/human-wt/ITA/PG01/2011/G2P4) was also sequenced, and used for comparison in each phylogenetic tree. RVA/human-wt/ITA/PG01/2011/G2P4 showed the same genotype as CEC06/2011 for VP6 and the NSPs (G2-P[4]-I2-A2-N2-T2-E2-H2). Obviously, it clustered as an outgroup in the VP7 and VP4 trees, and also showed major difference with the G6P[9] strain PG05/2011 for all the genes grouped in genotype 3 clusters. In the NSP1, NSP3, and NSP5 trees, the G2P[4] strain PG01/2011 showed strong similarity (99%) with G6P[6] strain CEC06/2011; conversely, in trees corresponding to VP6, NSP2, and NSP4, strain PG01/2011 clustered separately from strain CEC06/2011 (96%, 90%, and 92% similarities), while showing close relatedness to strains GER1H-09 [Pietsch et al., 2009], DRC86 and DRC88 [Matthijnssens et al., 2006] (Fig. 1a–h).

The amino acid homology of the VP8* gene of G6P[6] (CEC06/2011) and G6P[9] PG05/2011 strains was investigated with respect to the reference P[6] and P[9] strains recently shown to bind specific carbohydrates involved in cell binding [Hu et al., 2012; Huang et al., 2012]. The G6P[6] strain CEC06/2011 shared an overall similar VP4 amino acid sequence

with the reference P[6] RVA strains, and in particular it shared identical I101, D187, Y188, S189, S190, and T191 amino acids (numbering based on RRV VP8* sequence AY033150.1), which are residues involved in the rotavirus VP8* binding cleft. However, the Italian strain presented a deletion at position 134. In the same cleft region, the G6P[9] strain PG05/2011 showed four different amino acids out of six (R101, S187, Y188, Y189, L190, T191) with respect to the G6P[6] CEC06/2011 strain, whereas it shared the same residues identified in the G6P[14] strain HAL1166, identified originally in 1994 in Finland [Gerna et al., 1994], and reported recently to bind A-type histo-blood cellular antigens [Hu et al., 2012].

DISCUSSION

This study represents the first report in Italy, and the second in Europe [Matthijnssens et al., 2008b], of a human G6P[6] RVA strain, whereas G6P[9] RVA has previously been reported sporadically in Italy, having caused four infantile GE cases between 1986 and 2003 in Sicily [De Grazia et al., 2011]. Nonetheless, strain PG05/2011 represents the first case of a G6P[9] genotype circulating in the whole of peninsular Italy.

No other similar genotypes were found from any other children with RVA GE in the same areas in 2011, where most of patients were infected with G1P [8] rotaviruses or, to a lesser extent, with other common G/P genotypes (not shown).

Results of the phylogenetic analysis refute the possibility that the two Italian G6 strains could

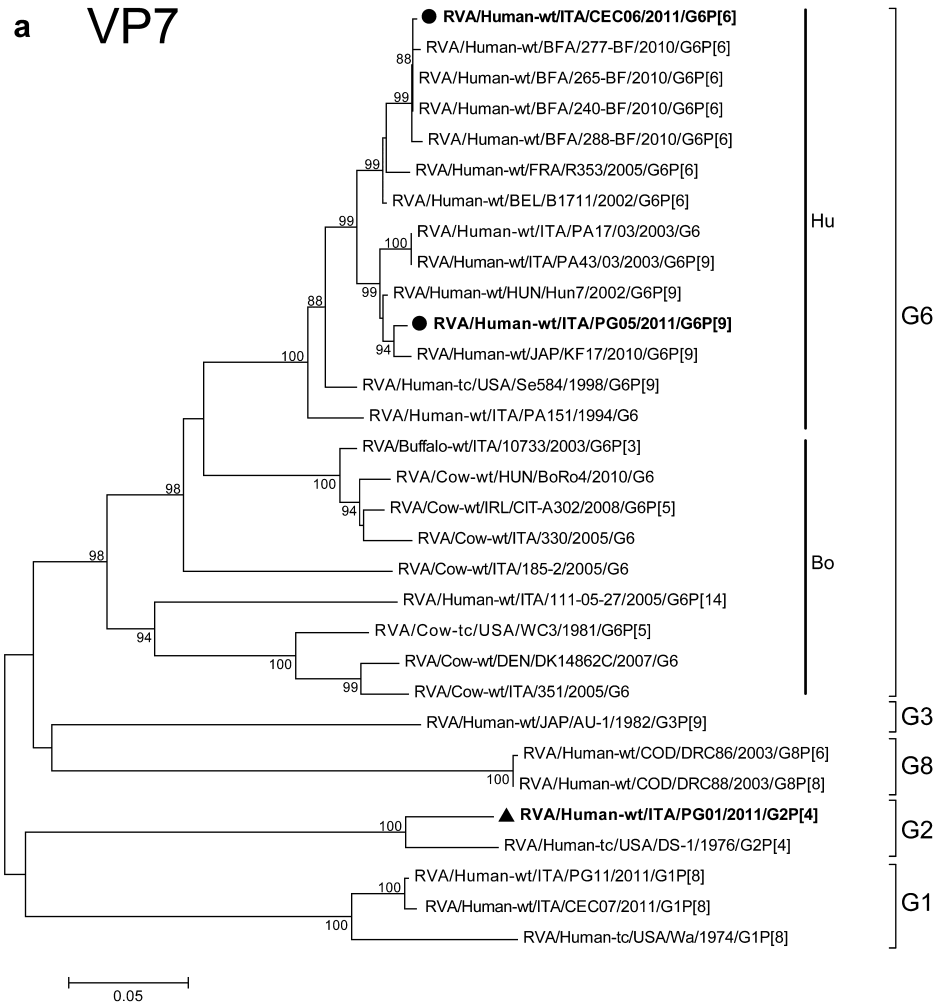


Fig. 1. Phylogenetic trees based on the nucleotide sequence of the entire ORF of genes encoding for: (**a**) VP7, (**b**) VP4 (VP8*), (**c**) VP6, (**d**) NSP1, (**e**) NSP2, (**f**) NSP3, (**g**) NSP4, and (**h**) NSP5. G6 strains CEC06/2011 and PG05/2011 are marked with a filled circle; reference DS-1 like strain is marked with a filled triangle. Trees were built with the neighbor-joining method and bootstrapped with 1,000 repetitions; bootstrap values below 70 are not shown. The Kimura-2 substitution model was used, as suggested by the MEGA5 ModelTest for best-fit evolutionary model selection. The bar indicates nucleotide substitutions per site.

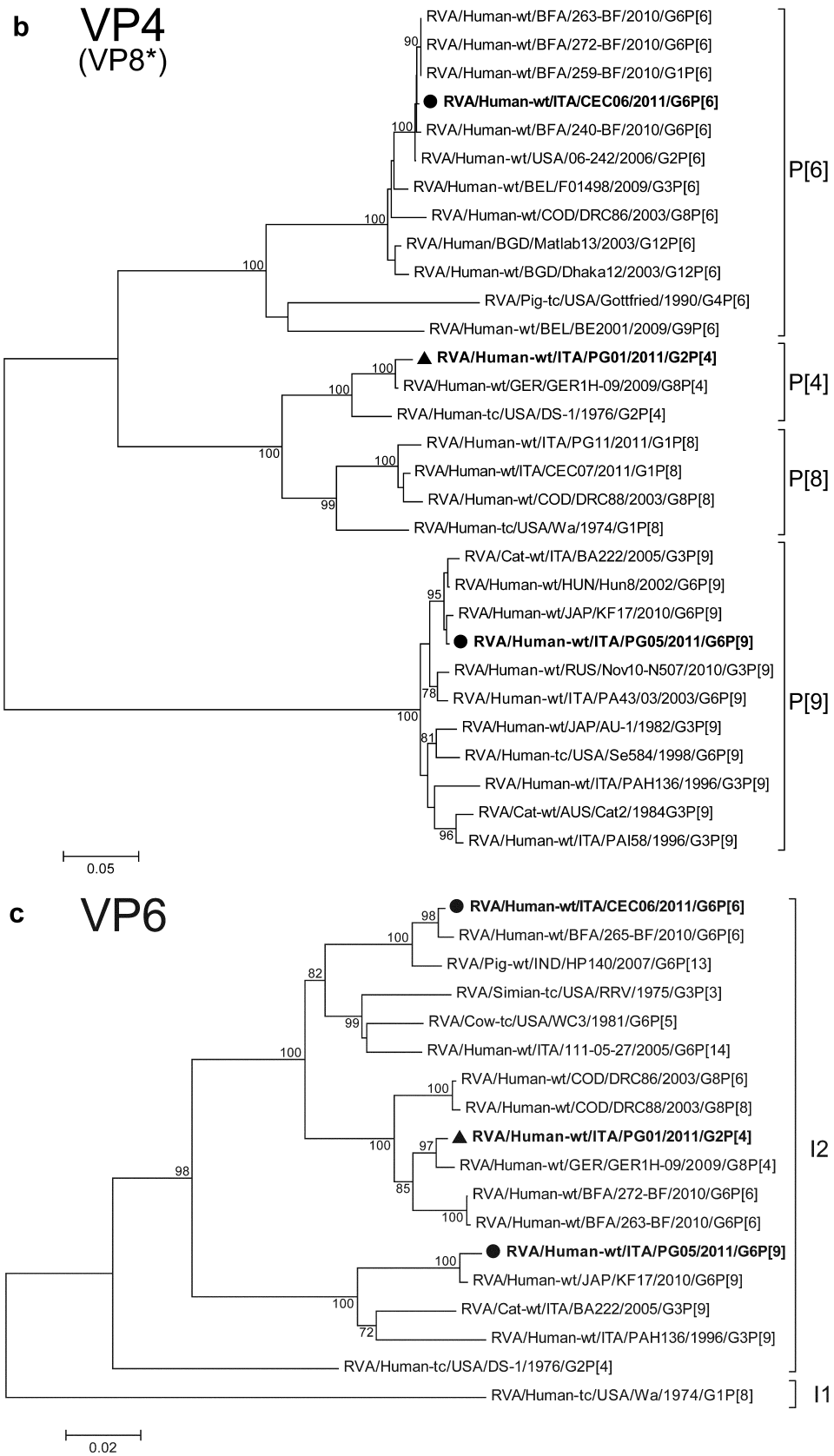
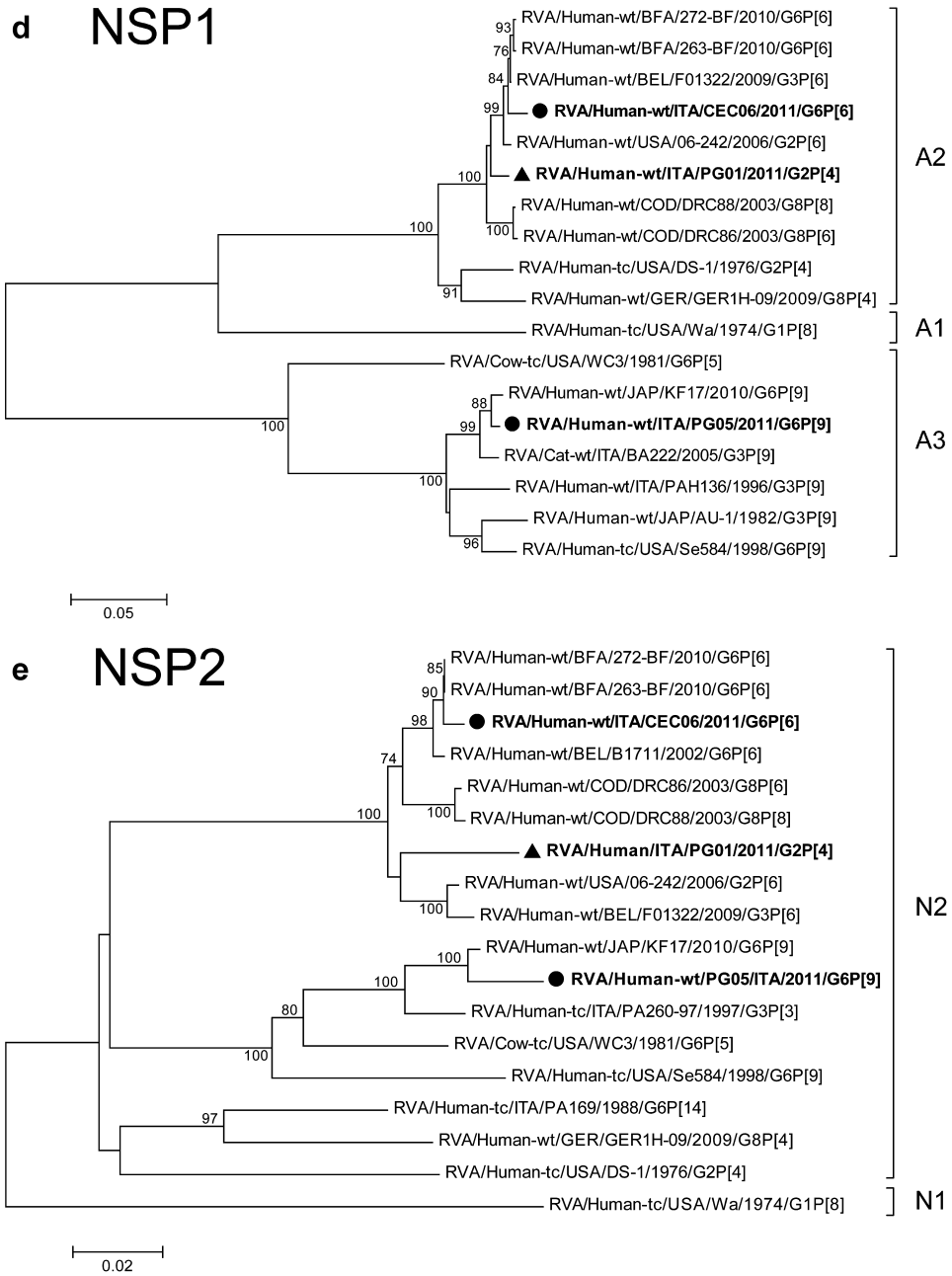


Fig. 1. (*Continued*)



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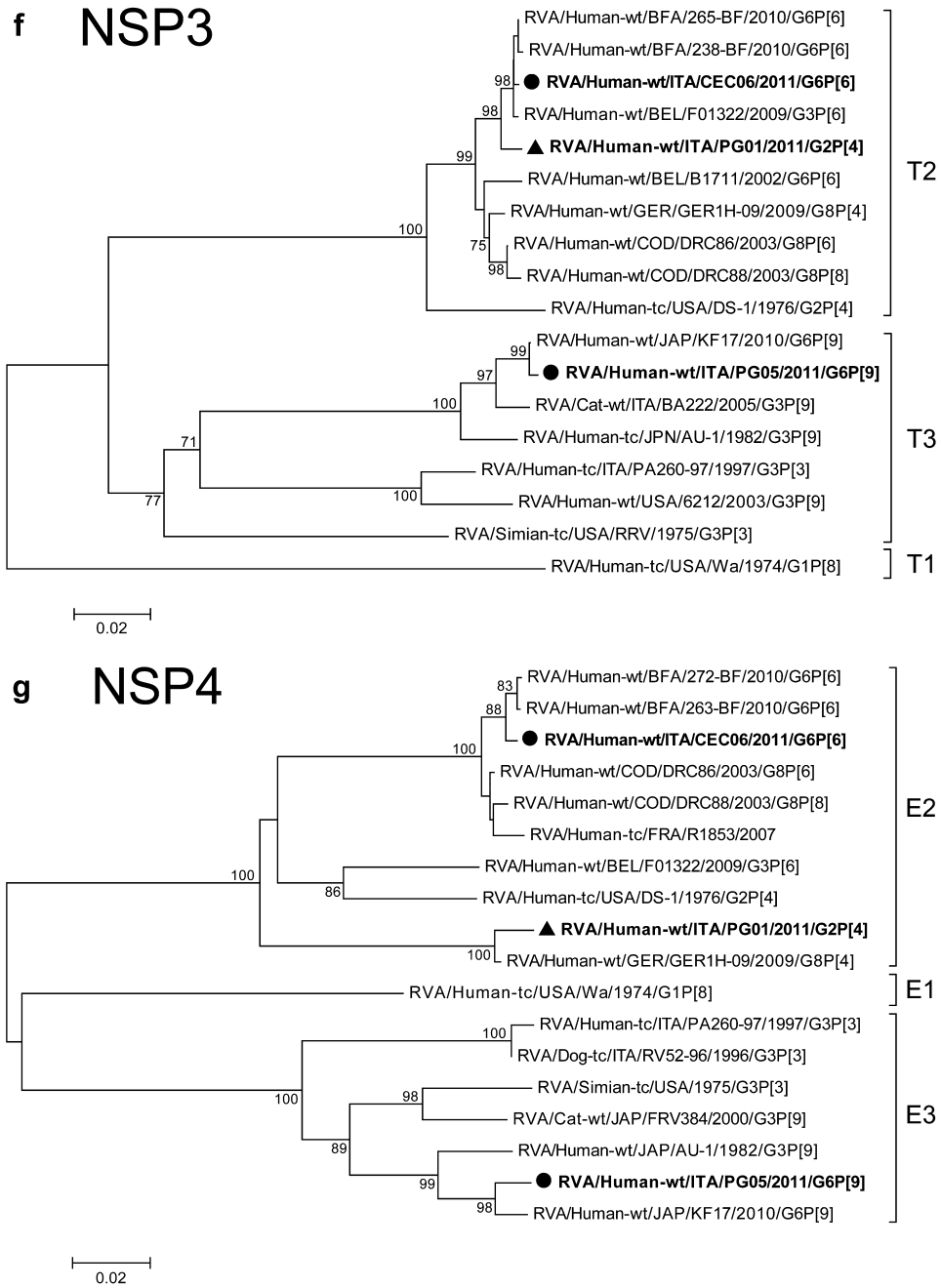
have originated from recent zoonotic reassortments between animal and human RVA strains. In fact, the VP7 G6 genotype of both strains CEC06/2011 and PG05/2011 corresponds closely to the G6 strains that circulated among humans during the last 10 years globally, and are probably derived from an earlier reassortment event with a bovine RVA donor.

Similar conclusions can also be reached from the analysis of the other genomic segments investigated, in that, the two Italian G6 strains reflect pre-existing RVA genotype constellations adapted to humans, not detected previously in Italy. Compared to a common DS-1 like G2P[4] RVA identified in the same area in 2011 (PG01/2011), it appears that although it shares a DS-1 like constellation strain CEC06/2011 represents a different rotavirus lineage, with marked differences particularly in VP6, NSP2, and NSP4 genomic segments. The G6P[9] strain PG05/2011 appears even more different in that it exhibited a mixed constellation not sharing any gene with the Italian G2P[4] RVA detected in the same city, during the same period.



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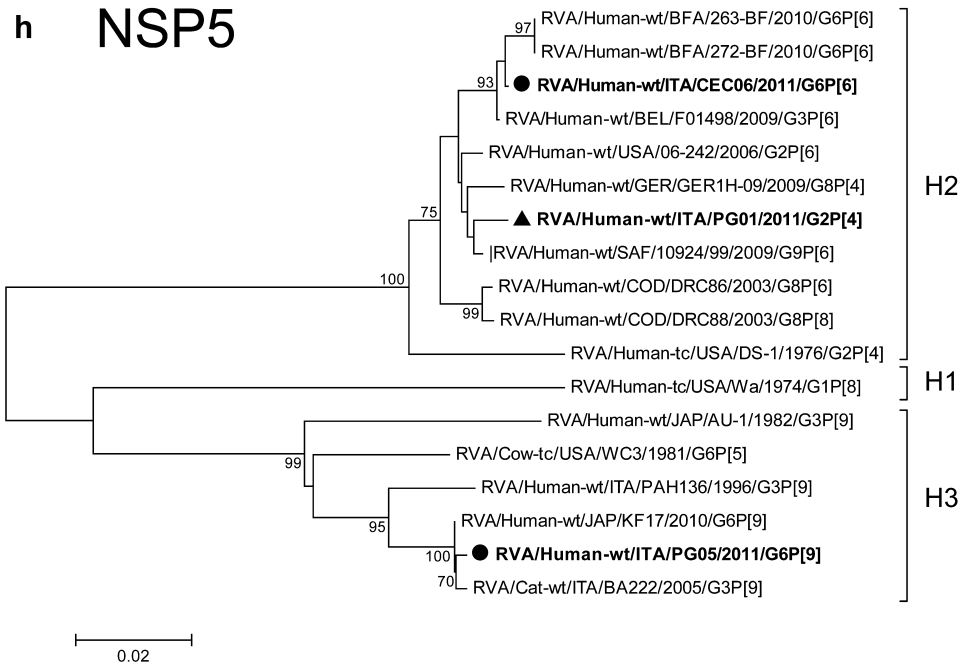
Compared to global strains, the G6P[6] strain CEC06/2011 was found to be very close to the G6P[6] viruses circulating in Burkina Faso in 2010 [Nordgren et al., 2012b], whereas the G6P[9] strain PG05/2011 was found to be closely similar to strain KF17, reported in Japan in 2010 [Yamamoto et al., 2011].

The detection of a G6P[6] strain in Italy is remarkable, considering that similar G6P[6] strains were responsible for 11% and 23% of the severe paediatric rotavirus GE cases, occurred in the urban and rural areas of a main town of Burkina Faso, respectively, resulting as the second most prevalent RVA genotype in that area [Nordgren et al., 2012a,b]. In light of its possible epidemic potential and because neither G6 nor P[6] are present in current vaccine formulations, it is obvious to argue whether similar emerging rotavirus strains would be contrasted effectively by either Rotarix® and Rotateq®.

The very high sequence similarity between strain CEC06/2011 and the G6P[6] RVA reported in Burkina Faso 1 year earlier [Nordgren et al., 2012b] suggests that this strain may have been imported to Italy



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as such. Nonetheless, it cannot be excluded that multiple reassortment events between variant genes of human and animal strains occurring sporadically in Italy may have also influenced the genome constellation of this specific strain, at least partially. The ill child infected with the G6P[6] CEC06/2011 strain was born from Senegalese parents, living within an African community in the city of Cecina. Although the patient had no history of travelling to Africa, it is possible that some members of the community have carried this RVA strain upon returning from their country of origin, eventually transmitting infection to surrounding citizens.

Strain PG05/2011 represents the second report worldwide of a G6P[9] genotype in association with an unusual mix of genomic constellations 2 and 3, the other strain having been reported recently in Japan [Yamamoto et al., 2011]. In both instances, RVA were detected in sporadic cases of disease, and were not related to epidemic outbreaks. The G6P[9] rotaviruses detected in Japan were suggested to represent a possible reassortment among uncommon bovine-like human RVA and human/feline AU-1-like RVA [Yamamoto et al., 2011]. Due to the quasi-identity between the Italian and Japanese strains in all eight genome segments sequenced, it is hard to believe that the G6P[9] strain PG05/2011 is an autochthonous strain generated in Italy. Rather, it is more likely that this strain was imported as such from Japan or that both the Italian and Japanese strains shared a common origin from a third country, events that should have occurred sufficiently close in time not to allow significant evolutionary divergence between the two strains.

The Italian patient infected with strain PG05/2011 lived in Perugia, which is renowned for the presence of a prestigious University open to foreigners. In fact, many Japanese students live in that town usually, and the G6P[9] virus might have been imported directly from Japan, possibly carried by an asymptomatic adult.

The amino acid sequence analysis of the VP8* region of the Italian G6P[6] and G6P[9] strains shows a marked difference between the two strains, as well as the strict similarity of these strains with other sialidase-insensitive P[6] or P[9] RVAs, respectively, investigated recently for the capacity to bind alternative cell receptors in a genotype-specific manner [Hu et al., 2012; Huang et al., 2012]. These researchers [Huang et al., 2012] proposed that P[6] rotavirus binding to H type 1 antigens could preferentially affect Afro Americans, among which they found this HBGA antigen to be particularly spread, based on previous unpublished studies. Although we cannot exclude that such a mechanism might also apply to the Italian G6P[6] strain, no information could be obtained regarding the actual expression of H1 antigen or the secretor status in the patient infected with this strain in Cecina. The patient infected with the G6P[9] RVA strain PG05/2011 belonged to the A-type histo-blood group, that was demonstrated to bind the VP4 of genotype P[9] as well as P[14] rotaviruses [Hu et al., 2012].

Overall, this study confirms that uncommon rotavirus strains can be introduced sporadically into a country, from remote areas of other continents. In contrast to the usual high rate of reassortment and/or genetic drift observed among rotaviruses co-circulating in a specific area, these unusual G6 RVA strains seem to be highly conserved, an observation that may either indicate a very recent importation from other countries or suggest that the maintenance of their overall gene constellation is necessary for them to infect humans efficiently. In addition, possible specific host genetic constraints influencing virus binding might limit spread of these viruses within smaller subpopulations, hampering further virus evolution. This might explain why no major spread of uncommon African rotavirus genotypes has been observed in Italy this far, despite the increasing international travelling and major immigration flows across the Mediterranean Sea.

Notwithstanding, the risk of importing novel RVA genotypes should not be neglected, particularly as RVA vaccination in some countries of Africa appears to be less efficacious than in industrialized countries [Madhi et al., 2010; Sow et al., 2012]. This may be related at least partially to the circulation of atypical genotypes in Africa, such as G6 and G8 in association with different P-types, not present in vaccines formulation. Together with other G and P types, the G6 RVA strains detected in Italy may thus represent a threat, and continuation of molecular surveillance and virus tracking programs may help prevent possible epidemic expansion of emerging strains.

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