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Detection and Molecular Characterization of Human Rotaviruses Isolated in Italy and Albania

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Rotaviruses are one of the most important causes of gastroenteritis in children under 5 years old. Analysis of G and P rotavirus genotypes in circulation is crucial in evaluating the appropriacy of mass vaccination of children worldwide. Overall, 592 stool samples were collected in Tirana (Albania), the Salento peninsula (South Italy), and three different hospitals in Rome (Central Italy). Of the total samples, 31.3% were rotavirus positive in Albania, 78.3% in the Salento, and 40.3% in Rome. The samples collected in Tirana and Rome were G–P typed, whereas the samples collected in the Salento were only G typed. Overall, in Italy the most frequent combinations were G4 P[8] (54.5%), G1 P[8] (27.3%), and G2 P[4] (18.2%); in Albania they were G9 P[8] (72.1%), G4 P[8] (8.8%), G1 P[8] (5.9%), and G2 P[4] (2.9%). The prevalence in Albania of atypical combinations was 7.4% for G4 P[4] and 2.9% for G9 P[4]. Phylogenetic analysis was also performed to assess the genetic relatedness of the strains. ***J. Med. Virol. 82:510–518, 2010.***

KEY WORDS: rotavirus; characterization; genotypes; co-circulation

INTRODUCTION

Rotaviruses are globally the leading cause of severe, dehydrating diarrhea in children aged <5 years, with an estimated 25 million outpatient visits and more than 2 million hospitalizations attributable to rotavirus infections each year [Parashar et al., 2006]. Annual fatalities in 2004 estimated to be 527,000 (475,000–580,000) occur predominantly in low-income countries [Anonymous, 2007]. In Europe, an estimated 3.6 million episodes of rotavirus gastroenteritis occur in children under 5 years old each year, leading to 700,000 outpatient visits, more than 87,000 hospital admissions and 231 deaths [Soriano-Gabarrò et al., 2006]. In Italy, rotaviruses are responsible for 26% of all cases of pediatric diarrhea and they are the most common pathogen, while 5–8% and 2% of cases are attributed to adenovirus and astrovirus, respectively [Marocco et al., 2006].

The rotavirus is a member of the *Reoviridae* family; the viral particles consist of three layers: the central core (enclosing 11 segments of double-stranded genomic RNA), the inner capsid, and the outer capsid [Van der Heide et al., 2005]. Rotavirus strains may be serotyped/genotyped on the basis of two outer capsid proteins that are the targets of neutralizing antibodies produced following natural infection: the glycoprotein VP7 and the protease-sensitive protein VP4. These two proteins form the basis of the present dual classification system of G (VP7) [Gouvea et al., 1990; Gilgen et al., 1997] and P (VP4) types [Gentsch et al., 1992; Das et al., 1994; Villena et al., 2003]. For G types a complete concordance of serotypes and genotypes has been achieved while for P types this is not the case. Accordingly, P genotypes are expressed with a "P" followed by a number in brackets (e.g., P[6]), whereas P serotypes are designated by "P" with a serotype number followed by the corresponding genotype in brackets (e.g., P2A[6]) [Desselberger et al., 2006]. Genotyping by reverse transcription-PCR (RT-PCR) has been used widely for detecting genetically distinct VP4 and VP7 genes.

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Although 14 G serotypes, 15 G genotypes, 14 P serotypes, and 27 P genotypes are known, only 11 G and 15 P genotypes have been identified in humans [Martinez-Laso et al., 2009]. The ability of rotavirus gene segments to segregate independently could readily lead to reassortment in doubly infected cells (as long as they are within the same A–G group), various combinations of proteins in progeny viruses have been observed, and correspondingly there is a great diversity of co-circulating wild-type rotavirus strains [Desselberger et al., 2006]. Epidemiological studies have indicated that there are four common rotavirus G types (G1–G4) [Martinez-Laso et al., 2009]. The G9 genotype has emerged as a causative agent of diarrhea in the United States, Canada, Australia, and several European, Latin American, African, and Asian countries [Ramachandran et al., 2000]. Other rare or uncommon rotavirus genotypes, such as G5, G8, and G12, have been detected in many countries across the world [Gouvea et al., 1994; Leite et al., 1996; Cunliffe et al., 2001; Le et al., 2008; Page et al., 2009]. There are two common P types (P[8] and P[4]), and one relatively uncommon P type (P[6]) [Martinez-Laso et al., 2009]. Currently, 5 G–P combinations (G1 P[8], G2 P[4], G3 P[8], G4 P[8], and G9 P[8]) cause approximately 90% of all human rotavirus infections in large areas of the world; type G1 P[8] is the most prevalent [Gentsch et al., 2005].

Rotavirus infections have been identified as an important target for vaccination. The first licensed vaccine was withdrawn [Van Damme et al., 2007] after it was shown to be associated with a risk of intussusceptions in infants. Two new live, oral, attenuated rotavirus vaccines were licensed in 2006: the monovalent human rotavirus vaccine (Rotarix™, GlaxoSmithKline, Verona, Italy) and the pentavalent bovine–human, reassortant vaccine (RotaTeq™, Merck, Milan, Italy). Both vaccines have demonstrated very good safety and efficacy profiles in large clinical trials in western industrialized countries and in Latin America [Vesikari et al., 2007]. This vaccine is focused on four G types (G1–G4) and one P type (P1A[8]) which account for 95% of the strains identified in humans globally. In trials, the vaccines provide 85–95% protection against infection severe enough to require hospitalization, and 72–74% protection against all rotavirus infection [Williams et al., 2009]. Surveillance and monitoring of rotavirus-related diseases, preferably through the establishment of sentinel surveillance sites, are essential for assessing the need for vaccination and the projected results of the vaccine in terms of reducing the burden of disease.

Albania is a Balkan country with a transition economy, which has considerable environmental, socio-economic, and health problems. In 1997, the country faced an outbreak of poliomyelitis involving more than 150 symptomatic subjects, and in 1994 there was a cholera outbreak. In recent years, in urban and rural areas around Tirana, the capital city of Albania, various outbreaks of hepatitis A and rotavirus have been recorded [Arrivi et al., 2007]. In this country, HAV is endemic and the risk of certain strains reaching Italy must be considered. The HAV18 strain, isolated from Italian environmental samples (personal data), showed complete homology with the ALB17 strain (accession number AY334031) (amino acid mutation, position 34 out of 56: substitution of Arg with Lys) identified in Albania [Gabrieli et al., 2004; Tamura et al., 2007].

Thus, the objectives of the present study were to determine the G and P genotypes of the rotavirus in circulation and to describe their genetic diversity; to verify possible co-circulation of viral strains between the areas and countries considered.

MATERIALS AND METHODS

Stool Sample Collection and Treatment

Overall, 592 stool specimens from children were collected in Italy and Albania. Specifically during the period May 2001 to April 2002, 313 stool samples were collected at the Paediatric and Emergency Units of Tirana University Hospital; between January 2004 and December 2005, 217 stool samples were collected in seven Hospitals in the province of Lecce, Italy (Casarano, Copertino, Galatina, Gallipoli, Lecce, Scorrano, and Tricase); between November 2006 and November 2007, 62 samples were collected in Rome, Italy, at the Paediatric Units of Sandro Pertini Hospital and S. Camillo-Forlanini Hospital. The samples were stored at −20°C until transfer to laboratories at Tor Vergata in Rome (Italy) (375 samples) and the University of the Salento (217 samples), for virological analysis by molecular biology techniques. Stools were diluted with phosphate-buffered saline (PBS) (Merck) or 0.89% NaCl to 10% suspensions. After vigorous mixing the fecal specimens were clarified by centrifugation at 2,500*g* for 20 min at 4°C, and then filtered using a 0.22-μm filter. The resulting supernatants were divided into aliquots and stored at −80°C until use for the detection of group A rotaviruses. Stool samples collected in Albania were tested previously for the enteric viruses as reported by Arrivi et al. [2007].

Viral Genome Extraction

Genomic viral RNA was extracted from 250 μl of 10% stool suspension, using the TRizol LS Reagent (Invitrogen, Milan, Italy) or the QIAamp Viral RNA kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. The final RNA pellet was resuspended in 55–60 μl of sterile water. Ten microliters was reverse transcribed immediately with specific primers, whereas the residual part was mixed with 1/10 of 5 M of NaCl, 2.5 volumes of pure ethanol and stored at −20°C.

Rotavirus Detection

Rotavirus infection was verified by means of rapid screening techniques currently available on the market, such as latex agglutination (Rho, Milan, Italy), and immuno-enzymatic (VIDAS Rotavirus, BioMerieux,

Florence, Italy) tests. According to the producers, these tests have a sensitivity and specificity of 95.7–100% and 96.3–100%, respectively. For generic detection of Group A rotaviruses, a RT-PCR method based on amplification of a VP6 fragment was used. Primers VP6-3 (5′-GCTTTAAAACGAAGTCTTCAAC-3′; position 2–23 of human strain Wa [accession number K02086]) and VP6-4 (5′-GGTAAATTACCAATTCCTCCAG-3′; position 187–166 of human strain Wa [accession number K02086]), each at a concentration of 1 μM, were used for the RT reaction in a 15-μl (final volume) mixture containing 4 U of AMV Reverse Transcriptase (Promega, Milan, Italy), 20 U of Recombinant RNasin Ribonuclease Inhibitor (Promega), each of the deoxynucleoside triphosphates at a concentration of 0.2 mM, and 10 μl of a denatured (5 min at 95°C) double-stranded RNA sample. The reaction mixture was incubated for 60 min at 42°C. Fifteen microliters of the RT product was processed by PCR using 2 U of GoTaq Flexi DNA Polymerase (Promega) in a 100 μl (final volume) mixture supplemented with both primers at a concentration of 0.5 μM, each of the deoxynucleoside triphosphates at a concentration of 0.2 mM and $MgCl_2$ at a concentration of 0.2 mM. The PCR program included a denaturation step at 95°C for 5 min and 40 amplification cycles for 1 min at 94°C, for 1 min at 50°C, and for 1 min at 72°C, followed by a final elongation step of 7 min at 72°C. The PCR product of 186 bp was loaded on 2% agarose gel in TAE buffer 1× with a 100-bp DNA ladder as a standard marker containing 0.5 μg of ethidium bromide per ml. After electrophoresis at 120 V for 1 hr, the gels were photographed under UV light. All the RT-PCR testing kits were obtained from Promega.

Rotavirus Genotyping by RT-PCR

Positive samples were tested further to determine G and P types using the methods described by Gouvea et al. [1990] and Gentsch et al. [1992], respectively. The cocktail of primers used in the multiplex reaction allowed determination of the G1, G2, G3, G4, G5, G8, and G9 VP7 types and the P[4], P[6], P[8], and P[9] VP4 types. Double-stranded RNA was first reverse transcribed and amplified by PCR with primer pairs corresponding to genes 4 and 7, 8, or 9 (depending on the strain), whose sequences are highly conserved. PCR typing was performed from dsDNA that was subsequently used as a template in the second amplification by using a cocktail of four (one for each of the gene 4 types considered) and seven (one for each of the gene 7 types considered) genetic group-specific minus-sense primers selected from gene sequences. The amplification products were analyzed by 2% agarose gel electrophoresis and visualized with UV after ethidium bromide staining. The G and P genotypes of the fecal samples were analyzed by comparing the size of the second-round PCR products with the amplification products of the relevant prototype reference strains. The sizes of typing bands for gene 4 types P[8], P[4], P[6], and P[9] were 345, 483, 267, and 391 bp, respectively, and for gene 7 types G5, G9, G3, G4, G2, G1, and G8 were 303, 306, 374, 583, 652, 749, and 885 bp, respectively.

For the G typing, a second set of primers was used: RV1 (5′-GTCACATCATACAATTCTAATCTAAG-3′; position 1061–1036 of human strain Wa [accession number K02033]), RV2 (5′-CTTTAAAAGAGAGAA-TTTCCGTCTG-3′, position 3–27 of human strain Wa [accession number K02033]), RV3 (5′-TGTATGGTATT-GAATATACCAC-3′, position 50–71 of human strain Wa [accession number K02033]), RV4 (5′-ACTGATCCT-GTTGGCCAWCC-3′, position 395–376 of human strain Wa [accession number K02033]) [Gilgen et al., 1997]. Double-stranded RNA was reverse transcribed with antisense primer (RV1). The completed RT reactions (20 μl) were processed first by amplification using 2 U of Go*Taq* Flexi DNA Polymerase (Promega) in a 100-μl (final volume) mixture supplemented with primers RV1 and RV2 at a concentration of 0.25 mM, each of the deoxynucleoside triphosphates at a concentration of 200 mM and $MgCl_2$ at a concentration of 1.5 mM. The PCR program included 1 min denaturation step at 94°C and 25 cycles of amplification for 30 sec at 94°C, for 60 sec at 55°C, and for 60 sec at 72°C, followed by a final elongation step of 3 min at 72°C. Two microliters of the first reaction were used as a template for the second round of PCR, using 0.5 mM of the second primer pair (RV3/RV4) and 3.5 mM $MgCl_2$. Cycling was done with 40 cycles of the same cycling profile of the first reaction (except for the extension step which was performed at 72°C for 10 min). The size of the bands was 346 bp. Aliquots of the second reaction (10 μl) were analyzed on 2% agarose gel in TAE buffer 1× with a 100-bp DNA ladder as a standard marker. The products were visualized by ethidium bromide staining and UV trans-illumination.

All the used primers were reported in Table I.

Phylogenetic Analysis of the Sequences

The amplified products were purified using the QIAgen PCR purification Kit (Qiagen) and sequenced using the same forward (RV3) primer. Sequence similarity was searched for with BLAST (www.ncbi.nlm.nih.gov/blast), multiple sequence alignment was conducted with ClustalW programs (www.ebi.ac.uk/clustalw) and phylogenetic analysis was performed on partial VP7 nucleotide sequences using version 4 of the MEGA software package [Tamura et al., 2007]. Genetic distances were calculated using the maximum likelihood algorithm. The dendrograms were constructed using the neighbor-joining method.

RESULTS

Overall, 592 stool samples were collected in Tirana (Albania), in the Salento area (South Italy) and in three different hospitals in Rome (Central Italy). Three hundred thirteen samples were collected in Albania during a 1 year study (May 2001 to April 2002) and 98 tested positive (31.3%). Two hundred seventeen samples were tested in the Salento area of which 170 tested

TABLE I. RT-PCR Primers

Primer	Sequence (5′–3′)	Map position	Amplicon (bp)	References
Rotavirus primers used for VP6 amplification				
VP6-3	GCT TTA AAA CGA AGT CTT CAA C	2–23[b]		
VP6-4	GGT AAA TTA CCA ATT CCT CCA G	187–166[b]	186	Villena et al. [2003a]
Rotavirus primers used for G-typing				
First round				
Beg9	GGC TTT AAA AGA GAG AAT TTC CGT TCG G	1–28[b]	1062	Gouvea et al. [1990]
End9	GGT CAC ATC ATA CAA TTC TAA TCT AAG	1062–1044[c]		Gouvea et al. [1990]
Second round				
G1	CAA GTA CTC AAA TCA ATG ATG G	314–335[b]	749	Gouvea et al. [1990]
G2	CAA TGA TAT TAA CAC ATT TTC TGT G	411–435[d]	652	Gouvea et al. [1990]
G3	CGT TTG AAG AAG TTG CAA CAG	689–709[e]	374	Gouvea et al. [1990]
G4	CGT TTC TGG TGA GGA GTT G	480–498[f]	583	Gouvea et al. [1990]
G5	GAC CTA ACA ACG AGT ACA TG		303	Gouvea et al. [1990]
G8	GTC ACA CCA TTT GTA AAT TCG	178–198[g]	885	Gouvea et al. [1990]
G9	CTA GAT GTA ACT ACA ACT AC	757–776[h]	306	Gouvea et al. [1990]
RVG-9	GGT CAC ATC ATA CAA TTC T	1062–1044[c]	1062	Gouvea et al. [1990]
Rotavirus primers used for P-typing				
First round				
Con-3	TGG CTT CGC TCA TTT ATA GAC A	11–32[i]		Gentsch et al. [1992, 2005]
Con-2	ATT TCG GAC CAT TTA TAA CC	868–887[i]	887	Gentsch et al. [1992, 2005]
Second round				
P[4]	CTA TTG TTA GAG GTT AGA GTC	474–494[j]	484	Gentsch et al. [1992, 2005]
P[6]	TGT TGA TTA GTT GGA TTC AA	259–278[k]	268	Gentsch et al. [1992, 2005]
P[8]	TCT ACT TGG ATA ACG TGC	339–356[i]	346	Gentsch et al. [1992, 2005]
P[9]	TGA GAC ATG CAA TTG GAC	385–402[l]	392	Gentsch et al. [1992, 2005]
Con-3	TGG CTT CGC TCA TTT ATA GAC A	11–32[i]		Gentsch et al. [1992, 2005]
Rotavirus primer used for VP7 amplification				
RV1	GTC ACA TCA TAC AAT TCT AAT CTA AG	1061–1036[b]		Gilgen et al. [1997]
RV2	CTT TAA AAG AGA GAA TTT CCG TCT	3–27[b]	1059	Gilgen et al. [1997]
RV3	TGT ATG GTA TTG AAT ATA CCA C	50–71[b]		Gilgen et al. [1997]
RV4[a]	ACT GAT CCT GTT GGC CAW CC	395–376[b]	346	Gilgen et al. [1997]

[a]W = A or T.
[b]VP6 amplification: referring to human strain Wa (serotype 1) [accession number K02033].
[c]HRV strain SA11 (serotype 3).
[d]HRV strain DS1 (serotype 2).
[e]HRV strain P(serotype 3).
[f]HRV strain ST3 (serotype 4).
[g]HRV strain 69M (serotype 8).
[h]HRV strain WI61 (serotype 9).
[i]HRV strain KU gene 4 sequence [accession number M21014].
[j]HRV strain RV5 [accession number M32559].
[k]HRV strain 1076.
[l]HRV strain K8.

positive (78.3%) but only 88 were randomly typed. Sixty-two samples were collected from sporadic cases in Rome and 25 (40.3%) showed a specific amplification band. The samples collected in Tirana and Rome were G–P typed, whereas the samples collected in the Salento were only G typed. Overall, 49.5% of the samples were scored positive by RT-PCR test.

Among the VP7 genotypes identified in Tirana, genotype G9 was predominant (66.3%) whereas G4 was identified in 15.3% of samples, G1 in 9.2% and G2 in 3.1%. Strains not G typed were 6 (6.1%). In Rome, the predominant G type was G4 (36.0%), G1 was observed in 32.0% of samples, G2 in 12.0%, and G9 in 8%. Three samples were not typed (12.0%). In the Salento area, the predominant genotype was G2 (40.9%), followed by G4 (22.7%), G9 (18.2%), G1 (15.9%), and G12 (2.3%) (tested only in the Salento area).

Among the VP4 genotypes (only Albanian and Rome), P[8] was the most frequent in Albania: 60.2% and in Italy 72.0%, P[4] was identified in 8.2% of the samples in Albania against 8.0% in Italy. Determination of the G–P combination types was possible only for samples collected in Albania and Central Italy. In Albania the combination G1 P[8] was identified in 5.9% of positive samples, G2 P[4] in 2.9%, G4 P[8] in 8.8%, and G9 P[8] in 72.1%. The prevalence of atypical combinations in Albania was 7.4% for G4 P[4] and 2.9% for G9 P[4]. In Central Italy, the most frequent combination was G4 P[8] (54.5%), the other combinations identified were G1 P[8] (27.3%) and G2 P[1] (18.2%) (Table II).

Tree Analysis

The G1 tree was divided into three clusters and the first one included strains from the two different geographical areas studied: Albania and Salento. In this cluster the homology of the various strains was between 97% and 99% with the strain PA9/03 with the exception of strain 277A with only 80% of nucleotide homology. The second cluster included seven strains (five Albanian and two from the Salento) with 98–99% homology with the strain chi-83 (135A, 258A, and 259A) and 98%

TABLE II. Rotavirus Genotypes in Albania, Central and South Italy

	Percentage and number of rotavirus identified		
	Albania	Rome (Italy)	Salento (Italy)
G genotype			
G1	9.2 (9)	32.0 (8)	15.9 (14)
G2	3.1 (3)	12.0 (3)	40.9 (36)
G4	15.3 (15)	36.0 (9)	22.7 (20)
G9	66.3 (65)	8.0 (2)	18.2 (16)
G12	0	0	2.3 (2)
Untypeable	6.1 (6)	12.0 (3)	0
Total % (n)	100.0 (98)	100.0 (25)	100.0 (88)
P genotype			
P1A[8]	60.2 (59)	72.0 (18)	Data not available
P1B[4]	8.2 (8)	8.0 (2)	Data not available
P3[9]	0	4.0 (1)	Data not available
Untypeable	31.6 (31)	16.0 (4)	Data not available
Total % (n)	100.0 (98)	100.0 (25)	
	Number of combinations of G–P		
	Albania	Rome (Italy)	Salento (Italy)
Combinations of G–P			
G1 P1A[8]	5.9 (4)	27.3 (3)	Data not available
G2 P1B[4]	2.9 (2)	18.2 (2)	Data not available
G4 P1B[4]	7.4 (5)	0	Data not available
G4 P1A[8]	8.8 (6)	54.5 (6)	Data not available
G9 P1A[8]	72.1 (49)	0	Data not available
G9 P1B[4]	2.9 (2)	0	Data not available

Albania: samples were collected between May 2001 and April 2002; Rome: samples were collected between November 2006 and November 2007; Salento: samples were collected between January 2004 and December 2005.

homology with the strain Mdv9816 (98A, 123A, and 68S); only 66S had 90% homology with Mdv9816. The third cluster was more heterogeneous including three reference strains, one Roman strain (P10) and the other group included only the Salento strains with 96–98% homology with CCH86 and 98% homology with DH402.

The G2 tree presented three clusters, the first one included only strains from the Salento area, one Albanian strain (303A). In this subcluster the homology was 97% with the international strain CCH248. The second cluster included only the Salento strains whereas the third cluster the Salento strains, two Albanian strains (138A and 284A) and the international reference strains isolated in China, Japan, Taiwan, and Bangladesh.

The G4 tree had a large cluster with strains from all the geographical area, a second cluster with Italian strains and one Albanian strain (123A). The G4 strain appeared more homogeneous than the other G types. The first cluster had 99% homology with the international strains included in the tree.

Finally, the G9 strain was the most abundant in Albania, found in 65 out of 98 positive samples and it was also identified in the Salento area but not in Central Italy. The G9 tree included, one large cluster with the most part of G9 isolated with 96–99% homology with the strain CIT254-RV and R2 with the exception of the strain 145A, 98% homology with the strain AHP52 isolated in Turkey. The second cluster presented only one Albanian strain (137A) with 97% homology with ITA-GUC49-2006. The third cluster included 7 international Italian strains, 10 isolated from the Salento area and 2 strains (P10 and P11) from Central Italy. One strain 60S had 94% homology with the Italian strain IT-MAR1-2005.

DISCUSSION

Human group A rotaviruses are the most predominant etiological agent in pediatric acute gastroenteritis as the Norovirus are the most predominant gastroenteritis viruses. In a recent study, it was calculated that 87,000 children were hospitalized, 700,000 were outpatients, and 231 died due to rotavirus-related gastrointestinal infection in European countries [Soriano-Gabarrò et al., 2006]. Analysis of circulating rotavirus genotypes (G and P strains) is the key to evaluating the suitability of mass vaccination of children worldwide. The observation of G and P distribution and diversity contribute to a better understanding of rotaviruses in circulation and help to characterize the various antigenic shifts that could reduce vaccine efficiency.

Recently, G9 rotavirus strains [Tcheremenskai et al., 2007] was identified at low rates in central-south eastern Europe, whereas in Italy this strain was first identified in humans in 1983 [Ansaldi et al., 2007]. In the present study, in Albania, they accounted for 66.3% of all positive samples and this correlates well with a previous research program. Villena et al. [2003b] found the most

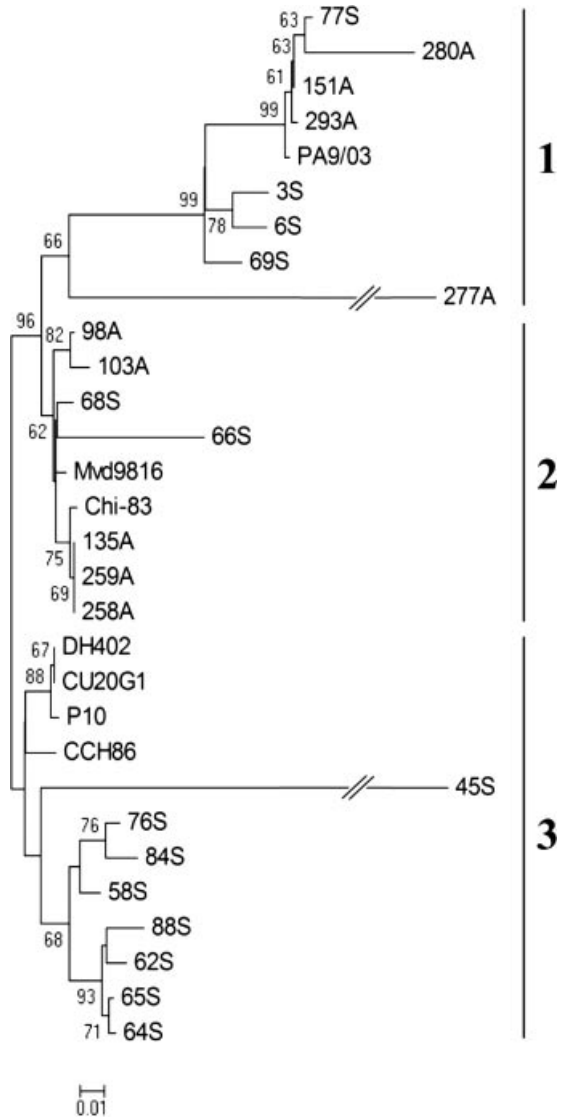

Fig. 1. Neighbor-joining phylogenetic tree based on partial VP7 nucleotide sequences of rotavirus genotype G1 strains. The numbers adjacent to the nodes represent the percentage of bootstrap support (of 500 replicates) for the clusters to the right of the node. Percentage bootstrap values above 50% are shown at the branch nodes. The tree including Italian strains isolated in different areas (F, Forlanin Hospital; P, Pertini Hospital, 1 sequences; S, Salento, 13 sequences; A, Albania, 9 sequences).

frequent combinations to be G9 P[8] and G3 P[8] accounting for 44% and 33% of outbreak samples, respectively, although the latter was not identified in the present study. G9 rotavirus strains are actually the fifth most common rotavirus isolated in the world [Ramachandran et al., 2000; Feeney et al., 2006]. Co-circulation of rotavirus strains in Albania and Italy, evident in the G1-G9 isolates (Figs. 1–4), can be explained by continuous population movement between two countries because of the low socio-economical conditions in Albania. Gabrieli et al. [2004] showed the co-presence of an HAV strain with the same amino acid mutations. Similarly, analysis confirms the

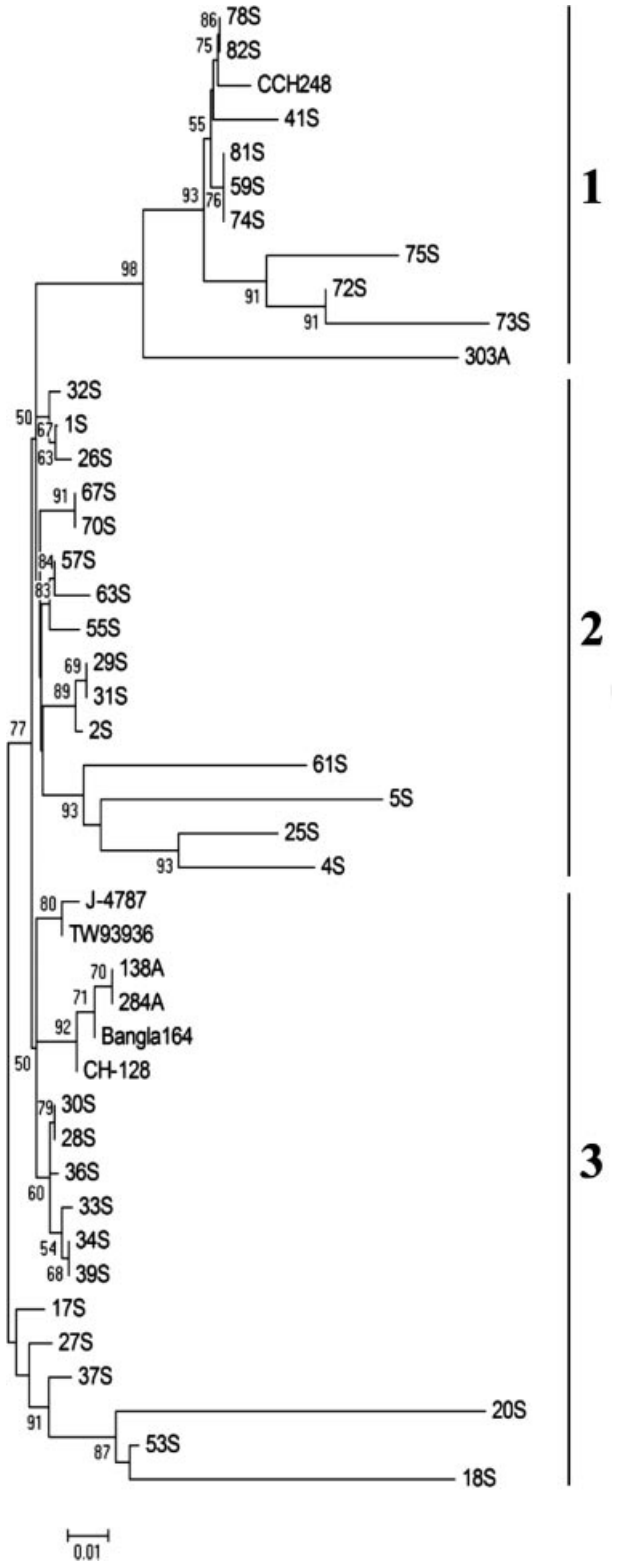

Fig. 2. Phylogenetic analysis of partial VP7 nucleotide sequences of serotype G2 strains (S, Salento, 36 sequences; A, Albania, 3 sequences).

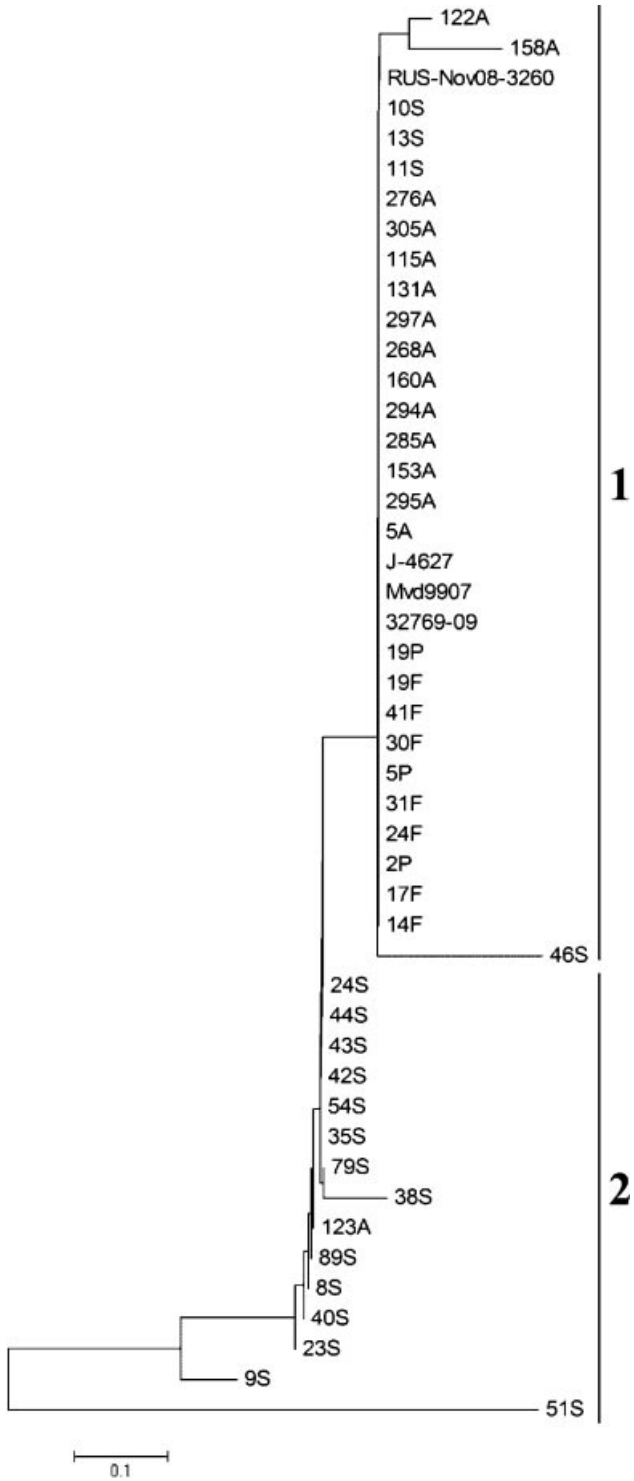

Fig. 3. Phylogenetic analysis of partial VP7 nucleotide sequences of serotype G4 strains (F, Forlanin Hospital; P, Pertini Hospital, 10 sequences; S, Salento, 18 sequences; and A, Albania, 15 sequences).

co-circulation of G1–G9 in Albania and Italy. For instance, for G9 in cluster 1, the homology was with the strains R2 and CIT-254RV, whereas all the international Italian strains are in the second and third cluster that includes only strains from South and Central Italy, showing a local circulation of these G9 strains.

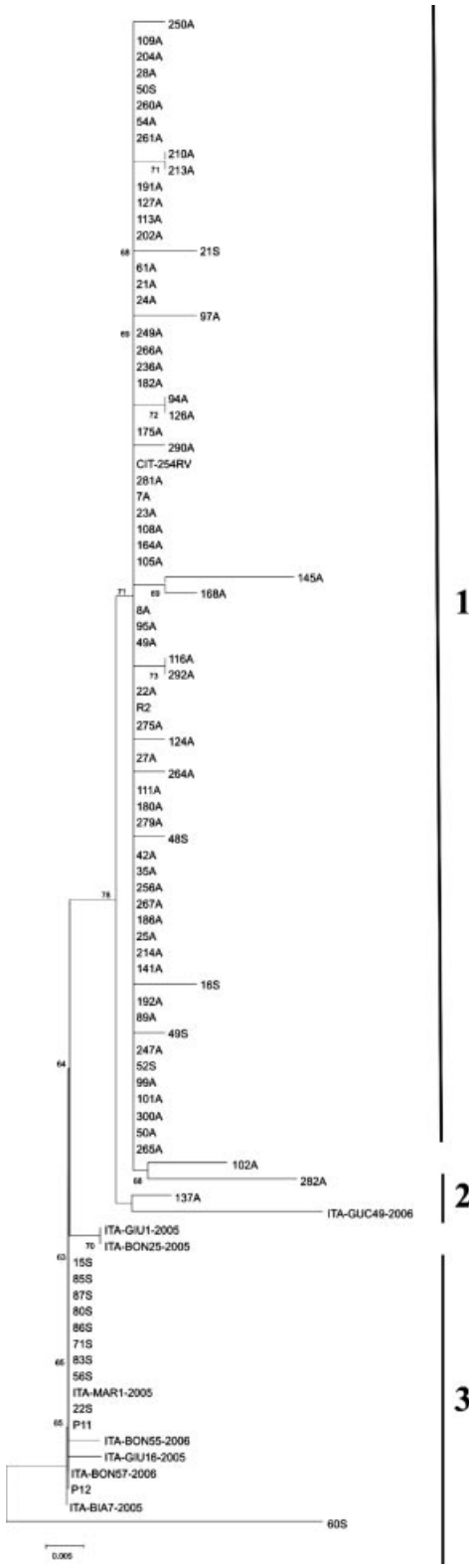

Fig. 4. Phylogenetic analysis of partial VP7 nucleotide sequences of serotype G9 strains (F, Forlanin Hospital; P, Pertini Hospital, 2 sequences; S, Salento, 16 sequences; A, Albania, 65 sequences).

The percentage of the G9 presence in the rest of Europe is very low going to 2.2% in Croatia to 11.1% in Slovenia. Only in Bulgaria this value is higher: 36.6%. The low value found in Albania can be explained with the different years of collection: 2004–2005 for Tcheremenskai et al. [2007] and 2001–2002 in the present study. We can suppose that the large circulation of this strain, viral gastroenteritis are the second cause of disease in children in Albania, and rotaviruses are the most common enteric virus [Arrivi et al., 2007], can have infected and protected the young people reducing the circulation of this genotype in 2004–2005. This G shift was reported by other authors [Buesa et al., 2000; Villena et al., 2003a] and confirmed by Tcheremenskai. The author found a genotypic shift from G9 to G4 in 2004 and G1 in 2005 in Albania. Martella et al. [2004], in the Salento region, in 2004 identified G9 strains at very high frequency (53.0%) more than the actual 28.1%.

The results of the present study confirm that all the rotavirus strains belonged to the most common G1–G4 types as observed in most part of the European Countries [Buesa et al., 2000; Villena et al., 2003a,b; Tcheremenskai et al., 2007]; besides, this study emphasizes the high frequency of P[8] strain [Arista et al., 1997; O'Mahony et al., 1999]. The second G type was the G4 in Albania and Rome but the G2 in the Salento. There may be different specific types of distribution in different areas as shown by Buesa et al. [2000] in the period 1996–1999 (shift from G4 to G1) in Valencia, whereas Villena [2003a], in Barcelona, in sewage samples, found a high presence of G1 (56.4%) and a low circulation of G9 (6%).

In Albania the principal combination of G and P is different from the other known results: G9 P[8] was identified in 72.1% of positive samples in the present study against 6.9% as reported by Tcheremenskai [2007].

The presence of unusual genotypes in Albania can be explained by natural circulation of these genotypes (which derive from rearrangement of rotavirus genes in highly endemic areas) or by the introduction of new variants due to the tendency to use the Balkan area as "entry" for the European Countries [Gabrieli et al., 2004; Tcheremenskai et al., 2007]. Unfortunately, the Salento isolates were not P typed and no unusual combinations were evident in Central Italy. This may depend either on the absence of unusual G–P types in Italy or on the low numbers of rotavirus-positive samples collected in Central Italy. However, studies conducted subsequently in the same area, the Salento area, highlighted the emergence of some novel strains [De Donno et al., 2009].

Mixed infections were present in Albania in less than 2% of all positive samples. Low values have also been identified in the United Kingdom [Iturriza-Gomara et al., 2000] and 6% in Spain [Sanchez-Fauquier et al., 2006]. Tcheremenskai et al. [2007], considering several countries from Central-South Europe, found mixed infections accounted for 12.7% of samples. This value is higher than the percentage found in this study but lower than the value found by Villena et al. [2003b] in an outbreak of rotaviruses in Albania.

The analysis of the G1–G9 trees clearly shows a low variability of the isolates. This can depend, as suggested by Arista et al. [2006] for human G1, by a lack of defined geographical pattern or by the political situation in Albania until the beginning of 1990s when the country was completely "closed." Gabrieli et al. [2004] analyzing the HAV circulation in Albania, confirmed the second hypothesis showing the presence of a unique genotype: IA. Another possibility is the stability of VP7 gene of rotavirus G1 as reported by Trinh et al. [2007] who found a substantial stability of G1 rotavirus isolated in Japan, China, Thailand, and Vietnam over a 10-year period.

When future vaccine will be designed, antigens from the different genogroups across the world should be included to ensure protection against all the strains. For this reason, the search for its G- and P-type distribution is necessary specially in given endemic region. On the contrary, it is also possible that the immunological pressure caused by vaccine might have resulted in the selection of new strains or cause an antigenic shift that could reduce in the years the protective efficiency of the vaccine.

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