



HAL
open science

Genetic characterization of G3 rotaviruses detected in Italian children in the years 1993-2005

Simona de Grazia, Vito Martella, Claudia Colomba, Antonio Cascio, Serenella Arista, Giovanni Maurizio Giammanco

► **To cite this version:**

Simona de Grazia, Vito Martella, Claudia Colomba, Antonio Cascio, Serenella Arista, et al.. Genetic characterization of G3 rotaviruses detected in Italian children in the years 1993-2005. *Journal of Medical Virology*, 2009, 81 (12), pp.2089. 10.1002/jmv.21615 . hal-00531824

HAL Id: hal-00531824

<https://hal.science/hal-00531824>

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



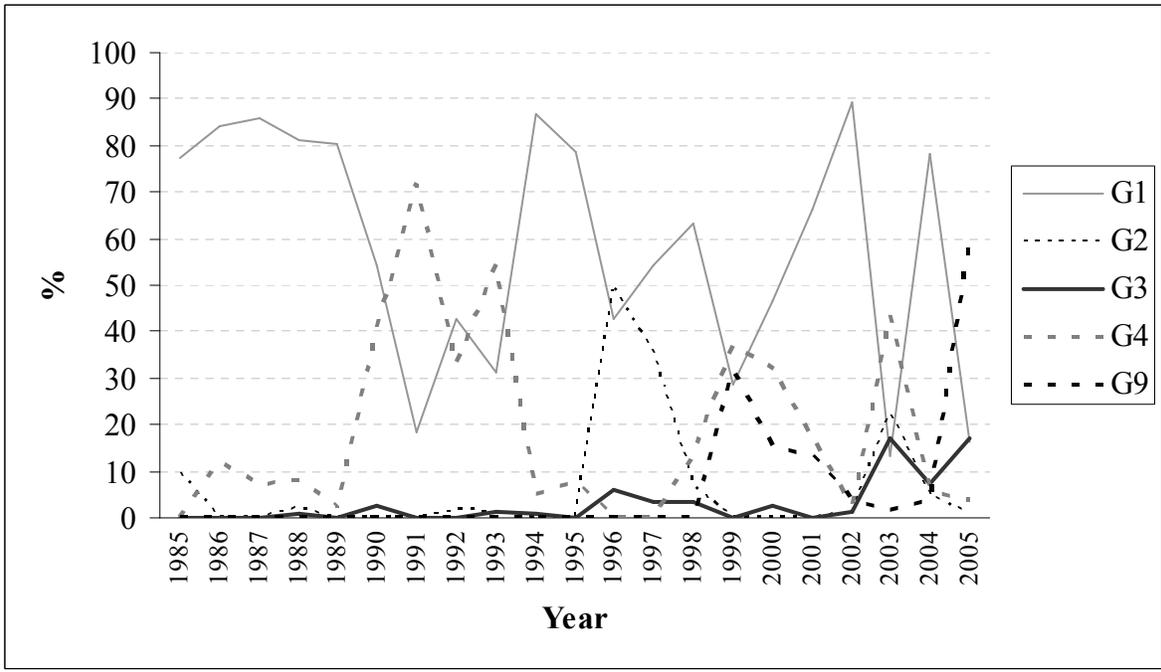
Genetic characterization of G3 rotaviruses detected in Italian children in the years 1993-2005

Journal:	<i>Journal of Medical Virology</i>
Manuscript ID:	JMV-09-1264.R2
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	26-Jun-2009
Complete List of Authors:	De Grazia, Simona; University of Palermo, Department of Health Promotion Sciences Martella, Vito; University of Bari, Public Health and Animal Sciences; University of Bari, Public Health and Animal Sciences Colomba, Claudia; University of Palermo, Department of Health Promotion Sciences Cascio, Antonio; University of Messina, Clinica delle Malattie Infettive Arista, Serenella; University of Palermo, Department of Health Promotion Sciences Giammanco, Giovanni; University of Palermo, Department of Health Promotion Sciences
Keywords:	rotavirus G3P[8], sequence analysis, NSP4, gastroenteritis, Italy



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 1



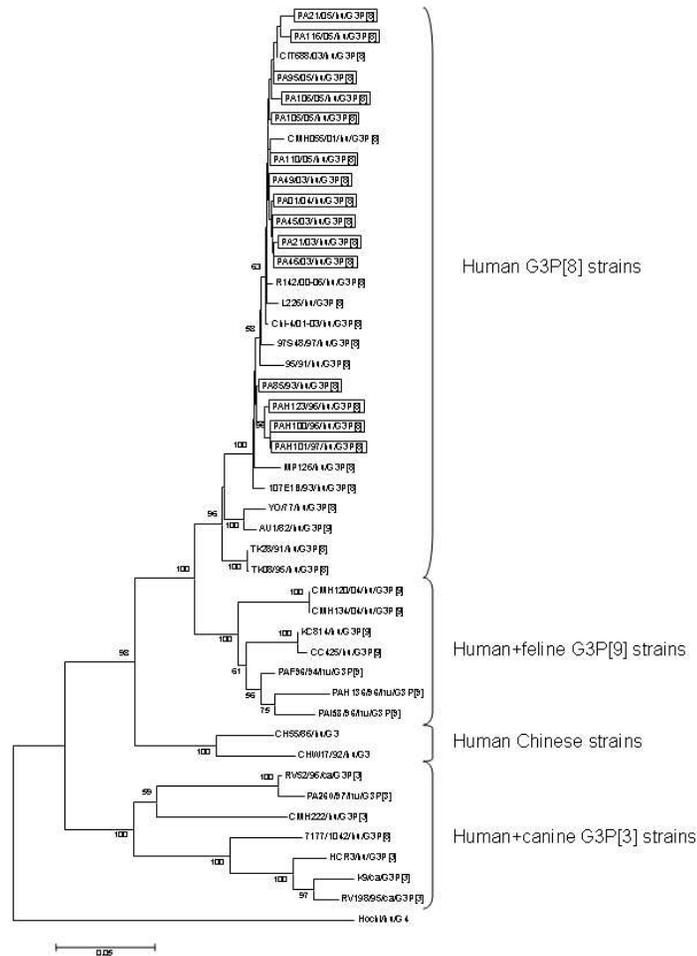


Figure 2. Phylogenetic analysis of nucleotide sequences of the VP7 gene of G3 strains. Italian G3P[8] strains are highlighted in boxes, G3P[9] and G3P[3] are in bold. The origin of rotavirus strains is indicated as follows: hu=human; ca=canine. The tree was generated based on the neighbor-joining method using ClustalW program. Bootstrap values above 50%, estimated with 1,000 pseudoreplicate data sets, are indicated at each node.

Figure 3

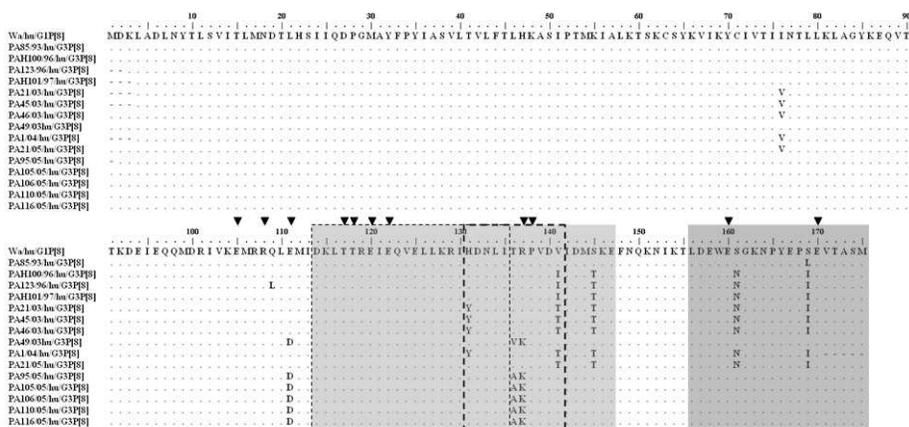


Figure 3. Deduced amino acid sequences of the NSP4 protein of Italian G3P[8] strains collected during the study period. The region involved in the binding to VP5* (aa 114-147) is indicated in light grey. The VP6-binding region (aa 156-175) is marked in dark gray. The interspecies variable domain (aa 131-141) is indicated by the thick dotted area. The sequence of the enterotoxigenic peptide (aa 114-135) corresponds to the thin dotted area. Critical residues for antibodies recognition are highlighted (▼). Conserved amino acid residues are indicated by dots. Wa/hu/G1P[8] is included as the NSP4 genotype E1 rotavirus standard.

275x190mm (96 x 96 DPI)

1
2
3
4 1 **Genetic characterization of G3 rotaviruses detected in Italian children in the**
5
6 2 **years 1993-2005.**
7

8
9 3 ¹Simona De Grazia, ²Vito Martella, ³Claudia Colomba, ⁴Antonio Cascio, ¹Serenella Arista,
10
11 4 ^{1*}Giovanni M. Giammanco
12

13
14 5
15
16 6 Dipartimento di Scienze per la Promozione della Salute “G. D’Alessandro”, Sezione di
17
18 7 Microbiologia¹, Sezione di Malattie Infettive³, Università di Palermo;
19
20 8 Dipartimento di Sanità Pubblica e Zootecnia, Università di Bari²;
21
22 9 Clinica delle Malattie Infettive, Università di Messina⁴
23
24

25 10
26
27
28 11 **Corresponding author:** Dipartimento di Scienze per la Promozione della Salute “G.
29
30 12 D’Alessandro”, via del Vespro 133, 90127, Palermo, Italia. Phone: +39 091 6553663; Fax: +39 091
31
32 13 6553676; e-mail address: g.m.giammanco@unipa.it (G.M. Giammanco).
33
34

35 14
36
37 15 **Shortened title:** Genotyping of Italian G3 rotaviruses
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 1 **Abstract**
4

5 2 In recent years an apparent increase in the frequency of detection of G3P[8] rotaviruses has been
6
7
8 3 observed worldwide. Similarly, in Italy G3P[8] strains have been detected sporadically and in a
9
10 4 scattered fashion over 20 years, whereas in 2003 and 2005 G3P[8] rotavirus activity increased
11
12 5 markedly. By analysis of the VP7, VP4, VP6 and NSP4 genes of a selection of G3P[8] rotaviruses
13
14
15 6 detected between 1993 and 2005, a remarkable sequence conservation was observed in the VP7,
16
17 7 VP4 and VP6 genes. By converse, after 2002 the Italian G3P[8] strains were found to possess
18
19
20 8 unique mutations in significant regions of the NSP4 protein.
21

22 9
23 10 **Keywords:** rotavirus G3P[8], sequence analysis, NSP4, gastroenteritis, Italy
24
25
26 11
27
28 12
29
30 13
31
32 14
33
34 15
35
36 16
37
38 17
39
40 18
41
42 19
43
44 20
45
46 21
47
48 22
49
50 23
51
52 24
53
54 25
55
56 26
57
58
59
60

1 Introduction

2 Rotaviruses are the major cause of acute gastroenteritis in children and infants worldwide,
3 being responsible for more than 600,000 deaths annually, chiefly in developing countries [Parashar
4 et al., 2006]. Rotaviruses, *Reoviridae* family, possess a triple-layered virion, that encloses 11 double
5 stranded (ds) RNA segments [Estes and Kapikian, 2007]. The two outer capsid proteins VP7 and
6 VP4 and the inner capsid protein VP6 are antigenically dominant and are used to classify rotavirus
7 strains into G (VP7) and P (VP4) types and to attribute the subgroup (VP6) specificity [Estes and
8 Kapikian, 2007]. To date, 22 G and 31 P genotypes, and 4 SG specificities have been established in
9 human and animal group A rotaviruses [Khamrin et al., 2006; Estes and Kapikian, 2007; Schumann
10 et al., 2009; Solberg et al., 2009; Trojnar et al., 2009]. Recently, based on sequence comparison, a
11 new classification system has been proposed for all the 11 genome segments [Matthijnssens et al.,
12 2008].

13 Although the genes coding for VP7, VP4, and VP6 segregate independently, and numerous
14 combinations are possible theoretically, many epidemiological studies indicated that G1, G3, G4
15 and G9 types are commonly associated with P[8] type, SGII specificity and a “long” pattern of
16 migration of the dsRNA segments in polyacrylamide gel electrophoresis (e-type), while G2 strains
17 display P[4] type, SGI specificity and “short” e-type [Estes and Kapikian, 2007]. The G1P[8]
18 rotavirus strains have been acknowledged universally as the most prevalent and ubiquitous, while
19 the incidence of strains with G2P[4], G3P[8], G4P[8] and G9P[8] specificities may vary regionally
20 and temporally. Other G/P combinations sporadically appear but may also acquire local relevance
21 [Santos and Hoshino, 2005].

22 The non-structural protein NSP4 is a key protein involved in morphogenesis and
23 pathogenesis of rotavirus [Estes and Kapikian, 2007]. Sequence analysis has revealed that the NSP4
24 gene of group A rotaviruses may be genetically classified into 11 genotypes, E1 to E11
25 [Matthijnssens et al., 2008], which include the five genotypes, Kun (A)-, Wa (B)-, Au-1 (C)-, EW
26 (D)-, or avian (E)-like, described previously. Within NSP4 genotypes E1 (B) and E2 (A), species-

1
2
3 1 specific patterns of segregation may be revealed by phylogenetic analysis [Ciarlet et al., 2000]. By
4
5
6 2 sequence analysis and PCR genotyping with specific primers, it has been shown that G1P[8],
7
8 3 G3P[8], G4P[8] and G9P[8] strains possess a NSP4 E1 (B) genotype, while G2P[4] have NSP4 E2
9
10 4 (A) genotype and G3P[9] viruses have either NSP4 E2 (A) or E3 (C) genotype [Estes and Kapikian,
11
12 5 2007; Bányai et al., 2009].

15 6 Rotavirus surveillance world-wide has shown in recent years changes in the distribution of
16
17 7 rotavirus G types, with decrease of G1 strains and parallel increase of G3 strains (15.8%-63%) in
18
19 8 Brazil, Japan, China, Russia, Ireland, Tunisia and Spain [Zhou et al., 2003; Fang et al., 2005; Lo et
20
21 9 al., 2005; Reidy et al., 2005; Sanchez-Fauquier et al., 2006; Yoshinaga et al., 2006; Chouikha et al.,
22
23 10 2007; Phan et al., 2007]. G3 rotaviruses infect almost all animal species and interspecies
24
25 11 transmission and/or reassortment with animal rotaviruses accounts likely for the detection of human
26
27 12 G3 strains with unusual genetic/antigenic features [Khamrin et al., 2006; De Grazia et al., 2007a;
28
29 13 De Grazia et al., 2008; Tsugawa and Hoshino, 2008].

34 14 In Italy, epidemiological surveillance for rotavirus enteritis in children has been carried out
35
36 15 uninterruptedly since 1985 in Palermo. The possibility to analyse a collection of rotavirus strains
37
38 16 spanning such a long time period has been useful for investigating the mechanisms of rotavirus
39
40 17 evolution [Arista et al., 2006]. In this study, a selection of G3 strains identified over a long temporal
41
42 18 range was analysed by comparing the genes coding for the outer capsid proteins VP7 and VP4, the
43
44 19 inner capsid protein VP6 and the non-structural protein NSP4.

1 **Materials and Methods**

2 **Samples.** In the period 1985-2006, 1591 rotavirus-positive samples were obtained from children of
3 less than 5 years of age, hospitalized with acute gastroenteritis at the “G. Di Cristina” Children’s
4 Hospital of Palermo. Fifteen of the 53 strains exhibiting a G3P[8] type were selected for this study
5 on the basis of the year of isolation, of the availability of faecal samples, and of RNA conservation,
6 as revealed by RT-PCR performed with multiple sets of specific primers (Table I).

7
8 **Determination of serotype and subgroup.** G typing was performed according to previously
9 published methods [Arista et al., 1990], with MAbs RV4:2, RV5:3, RV3:1, and ST3-3:1,
10 respectively, which are reactive with G1-to-G4-specific viral protein VP7 [Coulson et al., 1987].
11 Subgrouping was performed with subgroup I- and subgroup II specific MAbs (255/60 and 631/9)
12 reactive with viral protein VP6, as described elsewhere [Arista et al., 1990].

13
14 **RNA extraction and RT-PCR.** Rotavirus RNA was extracted from 10% fecal suspensions as
15 described by Boom et al. [Boom et al., 1990]. The extracted RNA was resuspended in RNase-free
16 sterile water and used for reverse transcription (RT)-PCR with random primers [Iturriza-Gòmara et
17 al., 1999]. To determine the G and P genotypes, specimens were analyzed with type-specific
18 primers by a heminested RT-PCR strategy. In a first PCR round, a 1,060-nucleotide-long fragment
19 of the gene encoding VP7 was amplified with a generic oligonucleotide primer pair (Beg9-End9)
20 [Gouvea et al., 1990; Iturriza-Gòmara et al., 2004], and the G types were subsequently predicted in
21 a second PCR round with a pool of internal primers specific for the G1, G2, G3, G4, and G9
22 genotypes in combination with the reverse consensus primer [Gouvea et al., 1990; Iturriza-Gòmara
23 et al., 2004]. Similarly, an 876-bp fragment of the fourth genome segment, encompassing the VP8*
24 portion of VP4, was amplified with generic primers Con3 and Con2, and P genotyping was carried
25 out with internal primers specific for the P[4], P[6], P[8], and P[9] genotypes [Gentsch et al., 1992;
26 Iturriza-Gòmara et al., 2004]. The PCR mixtures were prepared and thermal cycling was performed

1
2
3 1 as previously described [Iturriza-Gòmara et al., 2004]. Amplicons were analyzed by electrophoresis
4
5 2 with a 2% SeaKem LE (Cambrex Bio Science Rockland Inc., Rockland, ME) agarose gel in Tris-
6
7 3 acetate-EDTA at 6 V/cm for 60 min. To determine the E and I genotypes, the NSP4 and VP6 genes
8
9 4 were amplified by RT-PCR as described elsewhere [Lee et al., 2000; Iturriza-Gòmara et al., 2002].
10
11

12 5 **Sequence and phylogenetic analyses.** Amplicons of the genes encoding VP4, VP6, VP7 and NSP4
13
14 6 were directly sequenced with the specific primers. The amplicons were purified with GeneClean
15
16 7 purification spin columns (Q-biogene, Cambridge, United Kingdom) prior to sequencing with the
17
18 8 CEQ2000 Dye Terminator Cycle Sequencing Quick Start Kit (Beckman-Coulter, Fullerton, CA).
19
20 9 All methods were carried out by following the manufacturers' instructions. Sequences were
21
22 10 resolved with an automated sequencer (CEQ; Beckman-Coulter). Sequence alignment was
23
24 11 performed with CLUSTAL W [Thompson et al., 1994] and phylogenetic analysis was carried out
25
26 12 with the MEGA software, version 3.0 [Kumar et al., 2004], using the Kimura's two-parameter
27
28 13 distance model of correction and the neighbor-joining method to construct the phylogenetic tree.
29
30 14 The statistical significance of the inferred phylogenies was estimated by bootstrap analysis with
31
32 15 1,000 pseudoreplicate data sets. The sequences were aligned with and compared to additional
33
34 16 sequences obtained from online databases. Sequence alignment and phylogenetic analyses were
35
36 17 carried out on partial VP7 (904 bp), VP4 (VP8*) (480 bp), VP6 (316 bp) and NSP4 (717 bp)
37
38 18 fragments and on the deduced amino acids (aa).
39
40
41
42
43
44
45
46 19
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1 Results

2 In the surveillance period 1985-2006, among the 1591 rotavirus-positive samples obtained,
3 59 G3 rotaviruses (3.7%) were detected, with a high proportion (53/59=89.8%, or 3.3% of the
4 rotavirus-positive samples) of G3P[8] strains. G3 strains were detected at low prevalence (0-6.1%)
5 until 2002, whereas in 2003 and 2005 their prevalence reached 17% and 16.9%, respectively
6 (Figure 1). Fifteen of the 53 strains exhibiting a G3P[8] type were selected in order to investigate
7 their evolution (Table I). The VP7 serotype and the VP6 subgroup specificities of all of these strains
8 were initially determined with MAbs, and all of the strains exhibited G3 serotype and subgroup II
9 specificity. By sequence analyses of the VP6 gene, all the 15 G3P[8] strains were found to have a
10 genotype II, thus confirming the antigenic characterization with MAbs (data not shown). The
11 Italian G3P[8] strains displayed >85% nt and 93.3-100% aa identity to reference II strains in the
12 examined VP6 fragment. Nearly all of them tightly clustered with the reference II strain RMC437
13 (about 99% nt identity), but one strain, PA49/03, clustered with the II strain US0408 (99% nt
14 identity).

15 In the VP7 (nt 49-953)-based phylogenetic analysis (Figure 2), the Italian G3P[8]
16 rotaviruses clustered into a distinct lineage, along with the vast majority of the G3P[8] human
17 rotavirus strains, including the emerging G3 strains recently described in Japan, China, Russia,
18 Thailand and Ireland [Reidy et al., 2005; Phan et al., 2007; Trinh et al., 2007]. Almost all the human
19 Au-1-like G3P[9] strains were grouped in a distinct lineage, including also feline G3P[9] strains.
20 The Italian G3P[8] showed 97.4-99% aa identity to G3P[8] strains within the major lineage and
21 92.4-96.4% aa identity to human/feline G3P[9] strains. A third lineage included animal and animal-
22 like human G3 strains, likely derived from the canine host [De Grazia et al., 2007a]. A fourth
23 lineage exclusively included two Chinese G3 human rotaviruses isolated in 1986 and 1992.
24 Alignment of the deduced aa sequences of the antigenic variable regions (VRs) of VP7 [Estes and
25 Kapikian, 2007] showed no significant substitutions in VR7 (aa 141-150; antigenic region B), VR8
26 (aa 208-224; antigenic region C) and VR9 (aa 235-242; antigenic region F), while in VR5 (aa 87-

1
2
3 1 100; antigenic region A) a change at position 92 (Glu→Lys/Gln) was detected in one 1996 and in
4
5 2 two 2005 strains. An additional change, 91-Thr→Asn, was present in the VR5 of one of the two
6
7 3 2005 strains. Scattered aa mutations were also detected in the VRs 2, 3 and 4 of the VP7 of the
8
9 4 Italian G3P[8] strains (data not shown).

10
11
12 5 In the VP8* tree (data not shown), all the Italian G3P[8] rotaviruses clustered into the P[8]-
13
14 6 III lineage (95.1-100% of aa identity), according to the four-lineages (I to IV) scheme described
15
16 7 previously [Wen et al., 1997; Suzuki et al., 1998]. In the same lineage there were also the G1P[8],
17
18 8 G4P[8] and G9P[8] strains circulating in Palermo in the same years.

19
20
21
22 9 Upon sequence analysis, all the 15 Italian strains were characterised as NSP4 E1 (B)
23
24 10 genotype. The nt and aa identities to the prototype E1 strain Wa ranged from 92.9 to 99.7% and
25
26 11 from 96.3 to 99.4%, respectively. However, when the NSP4 aa sequences of the Italian G3P[8]
27
28 12 strains were analysed in detail (Figure 3), most of the 2003 isolates and the 2004 strain showed two
29
30 13 aa substitutions (131-His→Tyr and 141-Ile→Thr) within a region of the enterotoxin NSP4 with
31
32 14 demonstrated VP5*-binding activity (aa 114-147), whereas the oldest Italian G3P[8] strains did not.
33
34 15 In the same region, all the 2005 strains but one presented four or five aa substitutions (at position
35
36 16 131, 136, 137, 141 and 145) with respect to the earlier Italian G3P[8] strains. Only a single 2003
37
38 17 strain, PA49/03/hu/G3P[8], displayed the same mutations as the 2005 strains, with the exception of
39
40 18 position 136 where a different change (Thr→Val) was observed. In addition, the two most recent
41
42 19 VP5*-binding-region patterns were characterised by changes at position 111 and 137, that are
43
44 20 regarded as critical for antibody binding [Hyser et al., 2008], and by reversions at position 161 and
45
46 21 169 to the original Wa-pattern within the VP6-binding region (aa 156-175). Most of the punctuate
47
48 22 mutations observed in the Italian strains occurred in the interspecies variable domain (aa 131-141),
49
50 23 that has been associated with species-specific antibody response [Yuan et al., 2004].
51
52
53
54
55
56
57
58
59
60

1 Discussion

2 In the infant population of Palermo between 1985 and 2004, G2P[4], G4P[8], and G9P[8]
3 strains varied in their distribution or completely disappeared for short or long periods of time, while
4 G1 strains tended to circulate continuously (Figure 1) [De Grazia et al., 2007b]. By converse, the
5 prevalence of G3P[8] strains has always been very low, with the exception of the years 2003 and
6 2005, when their prevalence increased up to 17%. Previous studies have reported that G3
7 rotaviruses are intra-serotypically more heterogeneous than G1, G2 and G4 strains [Wen et al.,
8 1997; Suzuki et al., 1998] and this is likely accounted for by their broad host range. However, by
9 phylogenetic analysis of G3 human and animal viruses, it is possible to identify species-specific
10 patterns of segregation that are helpful to track the origin of animal-like or animal/human
11 reassortant strains [Nishikawa et al., 1989; Martella et al., 2001; 2008]. Human G3 rotaviruses are
12 usually associated with P[8] VP4 specificity, and only sporadically with a P[9] type. While the VP7
13 gene of P[8] strains markedly differs from the VP7 gene of P[9] viruses [De Grazia et al., 2008], the
14 VP7 sequences of G3P[8] strains of global origin have revealed that common human G3P[8] form a
15 homogenous group [Martella et al., 2008]. In this study, comparative sequence analysis of Italian
16 G3P[8] strains collected over a large time span provided further evidence for a continuous
17 remarkable conservation in the VP7. By converse, analysis of G1, G2, G4 and G9 strains circulating
18 during the same period in Palermo, Italy, depicted a different situation, with the appearance and
19 disappearance of several lineages and/or variants over the years [Arista et al., 2004; 2005a; 2005b;
20 2006]. In particular, the analysis of G1P[8] strains, the predominant rotavirus strains in Italy and
21 worldwide, revealed an highly heterogeneous viral population exhibiting multiple amino acid
22 changes in the VP7 and VP4 antigenic regions involved in rotavirus neutralization compared to
23 those circulating in the previous years, thus suggesting mechanisms of antigenic escape [Arista et
24 al., 2006]. This model may not apply to viruses that circulate at very low frequency in a given
25 population, such as G3P[8] rotaviruses. Interestingly, the Italian G3P[8] strains resembled in their
26 VP7 and VP4 genes G3P[8] rotavirus strains detected recently in Japan, China, Russia and Ireland

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
26

1 and reported to circulate at unusually high frequencies [Reidy et al., 2005; Phan et al., 2007; Trinh
2 et al., 2007]. Whether the increased activity of such G3P[8] strains relies on intrinsic features of the
3 VP7 and VP4, such as enhanced affinity for receptors or capsid stability, or on the properties of
4 other viral proteins, is unclear.

5 The NSP4 is a key protein involved in morphogenesis and pathogenesis of rotaviruses.
6 NSP4 acts as intracellular receptor for single-shelled particles and mediates viral assembly into
7 double shelled particles. NSP4 alone is able to induce diarrhea in experimental animals through a
8 Ca⁺⁺-dependent signalling pathway and this activity has been mapped to the enterotoxigenic
9 domain (aa 114 to 135). Antibodies against NSP4, or against the toxic peptide, are able to prevent
10 watery diarrhea, although the precise “in vivo” mechanisms of protection are unknown [Ball et al.,
11 2005; Estes and Kapikian, 2007] and the NSP4 is currently a target for vaccine development [Yu
12 and Langridge, 2001; Choi et al., 2006]. The analysis of the NSP4 gene of the G3P[8] Italian
13 isolates allowed their inclusion into genotype E1 (B), a finding that was not unexpected, due to the
14 strength of the genetic linkage within each of the three human genogroups (Wa-, DS-1- and AU-1-
15 like) [Bányai et al., 2009]. However, in the G3P[8] strains circulating in Palermo during 2003-2005,
16 multiple NSP4 amino acid substitutions were detected with respect to strains circulating in the
17 former years. These mutations affected the NSP4 region interacting with the VP4 (aa 114–147)
18 [Hyser et al., 2008] that includes the interspecies variable domain (aa 131-141) [Yuan et al., 2004]
19 and the sequence of the enterotoxic peptide (aa 114-135). Significant aa changes were observed also
20 at residues considered to be critical for antibody binding to NSP4 epitopes (residues 111 and 137)
21 [Hyser et al., 2008] and located within the VP6-binding region (residues 161 and 169) (Figure 3).
22 Whether these punctuate mutations may account for increased virus fitness, influenced by the
23 evolution of the NSP4-VP5* and/or NSP4-VP6 binding domains, or the appearance of NSP4
24 variants may facilitate the escape from acquired immunity is an intriguing possibility and surely
25 deserves attention. The NSP4 aa pattern encountered in most of the 2005 Italian strains is not
26 completely novel, since it has already been described in a G3P[8] strain collected in Leeds (UK) in

1
2
3 1 1999 [Iturriza-Gòmara et al., 2003]. In addition, Brazilian G1P[8] and G9P[8] strains with NSP4
4
5
6 2 patterns resembling the 2003 and 2005 G3P[8] NSP4 patterns have been detected in 2001, 2003 and
7
8 3 2004 [Araujo et al., 2007; de Moraes Tavares et al., 2008].
9

10 4 In conclusion, analysis of G3P[8] strains detected in Palermo, Italy, over nearly 20-years of
11
12 5 uninterrupted rotavirus surveillance revealed that these rotavirus strains tended to be highly
13
14 6 conserved. The low heterogeneity of G3P[8] rotaviruses could be accounted for by the observed
15
16 7 low prevalence in the infantile population, preventing the onset of immune-pressure mechanisms.
17
18 8 The apparent increase in G3P[8] activity observed in Italy and in other countries worldwide remains
19
20 9 unclear. However, in this study some aa mutations in significant regions of the NSP4 protein
21
22 10 allowed to differentiate the old strains from the recent G3P[8] viruses, suggesting a possible
23
24 11 correlation with the G3P[8] peaks observed in 2003 and 2005. These findings may provide clues
25
26 12 for understanding of the mechanisms of evolution of rotavirus, which could be addressed in more
27
28 13 extensive investigations based on national and international surveillance programmes of rotavirus.
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1 **Acknowledgments**

2 This study was supported by the Ministero dell’Istruzione, dell’Università e della Ricerca (Italian
3 Ministry of Education, University and Reserch)(Fondi di Ateneo ex 60%).

4

For Peer Review

Table I. Distribution and typing of the 59 G3 rotavirus strains circulating in Palermo in the surveillance period 1985-2005.

Year	Total number of G3 rotavirus strains identified (%)	PAGE e-type	VP6 SG	G-serotype	P genotype	Strains included in this study	
1988	1(1)	long	I	G3	P[14]	-	
1990	2 (2.5)	long	II	G3	P[8]	-	
1993	1 (1.2)	long	II	G3	P[8]	PA85/93	
1994	1 (0.9)	long	II	G3	P[9]	PAF96/94*	
1996	6 (6.1)	4	long	II	G3	P[8]	PAH100/96, PA123/96
		2	long	I	G3	P[9]	PAH136/96*, PAI58/96*
1997	3 (3.5)	1	short	I	G3	ND	-
		1	long	II	G3	P[8]	PAH101/97
		1	long	I	G3	P[3]	PA260/97*
1998	1 (3.3)	long	II	G3	P[8]	-	
2000	3 (2.5)	long	II	G3	P[8]	-	
2002	1 (2.6)	long	II	G3	P[8]	-	
2003	9 (17)	long	II	G3	P[8]	PA21/03, PA45/03, PA46/03, PA49/03	
2004	6 (7.3)	long	II	G3	P[8]	PA01/04	
2005	25 (16.9)	long	II	G3	P[8]	PA21/05, PA95/05, PA105/05, PA106/05, PA110/05, PA116/05	
Total	59 (3.7)						

* These 4 strains having other than P[8] type have been described in details elsewhere [De Grazia et al., 2007a; 2008].

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
26**1 REFERENCES**

- 2 Araujo IT, Heinemann MB, Mascarenhas JD, Assis RM, Fialho AM, Leite JP. 2007. Molecular
3 analysis of the NSP4 and VP6 genes of rotavirus strains recovered from hospitalized
4 children in Rio de Janeiro, Brazil. *J Med Microbiol* 56(Pt 6):854-859.
- 5 Arista S, Giovannelli L, Pistoia D, Cascio A, Parea M, Gerna G. 1990. Electropherotypes,
6 subgroups and serotypes of human rotavirus strains causing gastroenteritis in infants and
7 young children in Palermo, Italy, from 1985 to 1989. *Res Virol* 141(4):435-448.
- 8 Arista S, Giammanco GM, De Grazia S, Migliore MC, Martella V, Cascio A. 2004. Molecular
9 characterization of the genotype G9 human rotavirus strains recovered in Palermo, Italy,
10 during the winter of 1999-2000. *Epidemiol Infect* 132(2):343-349.
- 11 Arista S, Giammanco GM, De Grazia S, Colomba C, Martella V. 2005a. Genetic variability among
12 serotype G4 Italian human rotaviruses. *J Clin Microbiol* 43(3):1420-1425.
- 13 Arista S, Giammanco GM, De Grazia S, Colomba C, Martella V, Cascio A, Iturriza-Gòmara M.
14 2005b. G2 rotavirus infections in an infantile population of the South of Italy: variability of
15 viral strains over time. *J Med Virol* 77(4):587-594.
- 16 Arista S, Giammanco GM, De Grazia S, Ramirez S, Lo Biundo C, Colomba C, Cascio A, Martella
17 V. 2006. Heterogeneity and temporal dynamics of evolution of G1 human rotaviruses in a
18 settled population. *J Virol* 80(21):10724-10733.
- 19 Ball JM, Mitchell DM, Gibbons TF, Parr RD. 2005. Rotavirus NSP4: a multifunctional viral
20 enterotoxin. *Viral Immunol* 18(1):27-40.
- 21 Bányai K, Bogdán A, Szücs G, Arista S, De Grazia S, Kang G, Banerjee I, Iturriza-Gòmara M,
22 Buonavoglia C, Martella V. 2009. Assignment of the group A rotavirus NSP4 gene into
23 genotypes using a hemi-nested multiplex PCR assay: a rapid and reproducible assay for
24 strain surveillance studies. *J Med Microbiol* 58:303-311.
- 25 Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. 1990.
26 Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 28(3):495-503.

- 1
2
3 1 Choi NW, Estes MK, Langridge WH. 2006. Mucosal immunization with a ricin toxin B subunit-
4
5 2 rotavirus NSP4 fusion protein stimulates a Th1 lymphocyte response. *J Biotechnol*
6
7
8 3 121(2):272-283.
9
- 10 4 Chouikha A, Fodha I, Noomen S, Bouzid L, Mastouri M, Peenze I, De Beer M, Dewar J, Geyer A,
11
12 5 Sfar T, Gueddiche N, Messaadi F, Trabelsi A, Boujaafar N, Steele AD. 2007. Group A
13
14 6 rotavirus strains circulating in the eastern center of Tunisia during a ten-year period (1995-
15
16 7 2004). *J Med Virol* 79(7):1002-1008.
17
18
- 19 8 Ciarlet M, Liprandi F, Conner ME, Estes MK. 2000. Species specificity and interspecies relatedness
20
21 9 of NSP4 genetic groups by comparative NSP4 sequence analyses of animal rotaviruses.
22
23 10 *Arch Virol* 145(2):371-383.
24
25
- 26 11 Coulson BS, Fowler KJ, White JR, Cotton RG. 1987. Non-neutralizing monoclonal antibodies to a
27
28 12 trypsin-sensitive site on the major glycoprotein of rotavirus which discriminate between
29
30 13 virus serotypes. *Arch Virol* 93(3-4):199-211.
31
32
33
- 34 14 De Grazia S, Martella V, Giammanco GM, Iturriza-Gòmara M, Ramirez S, Cascio A, Colomba C,
35
36 15 Arista S. 2007a. Canine-origin G3P[3] rotavirus strain in child with acute gastroenteritis.
37
38 16 *Emerg Infect Dis* 13(7):1091-1093.
39
40
- 41 17 De Grazia S, Ramirez S, Giammanco GM, Colomba C, Martella V, Lo Biundo C, Mazzola R,
42
43 18 Arista S. 2007b. Diversity of human rotaviruses detected in Sicily, Italy, over a 5-year
44
45 19 period (2001-2005). *Arch Virol* 152:833-837.
46
47
- 48 20 De Grazia S, Giammanco GM, Martella V, Ramirez S, Colomba C, Cascio A, Arista S. 2008. Rare
49
50 21 AU-1-like G3P[9] human rotaviruses with a Kun-like NSP4 gene detected in children with
51
52 22 diarrhea in Italy. *J Clin Microbiol* 46(1):357-360.
53
54
- 55 23 de Moraes Tavares T, Elsner Diederichsen de Brito WM, Sousa Fiaccadori F, Leal de Freitas ER,
56
57 24 Alves Parente J, Sucasas da Costa PS, Gimenes Giugliano L, Assis Andreasi MS, Almeida
58
59 25 Soares CM, de Paula Cardoso DD. 2008. Molecular characterization of the NSP4 gene of

- 1
2
3 1 human group A rotavirus samples from the West Central region of Brazil. Mem Inst
4
5
6 2 Oswaldo Cruz 103(3):288-294.
7
8 3 Estes MK, Kapikian AZ. 2007. Rotaviruses. Knipe DM, Howley PM, editors. Philadelphia:
9
10 4 Lippincott, Williams and Wilkins. 1917-1974 p.
11
12 5 Fang ZY, Wang B, Kilgore PE, Bresee JS, Zhang LJ, Sun LW, Du ZQ, Tang JY, Hou AC, Shen H,
13
14 6 Song XB, Nyambat B, Hummelman E, Xu ZY, Glass RI. 2005. Sentinel hospital
15
16 7 surveillance for rotavirus diarrhea in the People's Republic of China, August 2001-July
17
18 8 2003. J Infect Dis 192 Suppl 1:S94-99.
19
20 9 Gentsch JR, Glass RI, Woods P, Gouvea V, Gorziglia M, Flores J, Das BK, Bhan MK. 1992.
21
22 10 Identification of group A rotavirus gene 4 types by polymerase chain reaction. J Clin
23
24 11 Microbiol 30(6):1365-1373.
25
26 12 Gouvea V, Glass RI, Woods P, Taniguchi K, Clark HF, Forrester B, Fang ZY. 1990. Polymerase
27
28 13 chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. J
29
30 14 Clin Microbiol 28(2):276-282.
31
32 15 Hyser JM, Zeng CQ, Beharry Z, Palzkill T, Estes MK. 2008. Epitope mapping and use of epitope-
33
34 16 specific antisera to characterize the VP5* binding site in rotavirus SA11 NSP4. Virology
35
36 17 373(1):211-228.
37
38 18 Iturriza-Gòmara M, Green J, Brown DW, Desselberger U, Gray JJ. 1999. Comparison of specific
39
40 19 and random priming in the reverse transcriptase polymerase chain reaction for genotyping
41
42 20 group A rotaviruses. J Virol Methods 78(1-2):93-103.
43
44 21 Iturriza-Gòmara M, Wong C, Blome S, Desselberger U, Gray J. 2002. Molecular characterization of
45
46 22 VP6 genes of human rotavirus isolates: correlation of genogroups with subgroups and
47
48 23 evidence of independent segregation. J Virol 76(13):6596-6601.
49
50 24 Iturriza-Gòmara M, Anderton E, Kang G, Gallimore C, Phillips W, Desselberger U, Gray J. 2003.
51
52 25 Evidence for genetic linkage between the gene segments encoding NSP4 and VP6 proteins
53
54 26 in common and reassortant human rotavirus strains. J Clin Microbiol 41(8):3566-3573.
55
56
57
58
59
60

- 1
2
3 1 Iturriza-Gòmara M, Kang G, Gray J. 2004. Rotavirus genotyping: keeping up with an evolving
4
5 2 population of human rotaviruses. *J Clin Virol* 31(4):259-265.
6
7
8 3 Khamrin P, Maneekarn N, Peerakome S, Yagyu F, Okitsu S, Ushijima H. 2006. Molecular
9
10 4 characterization of a rare G3P[3] human rotavirus reassortant strain reveals evidence for
11
12 5 multiple human-animal interspecies transmissions. *J Med Virol* 78(7):986-994.
13
14
15 6 Kumar S, Tamura K, Nei M. 2004. MEGA3: Integrated software for Molecular Evolutionary
16
17 7 Genetics Analysis and sequence alignment. *Brief Bioinform* 5(2):150-163.
18
19
20 8 Lee CN, Wang YL, Kao CL, Zao CL, Lee CY, Chen HN. 2000. NSP4 gene analysis of rotaviruses
21
22 9 recovered from infected children with and without diarrhea. *J Clin Microbiol* 38(12):4471-
23
24 10 4477.
25
26
27 11 Lo JY, Szeto KC, Tsang DN, Leung KH, Lim WW. 2005. Changing epidemiology of rotavirus G-
28
29 12 types circulating in Hong Kong, China. *J Med Virol* 75(1):170-173.
30
31
32 13 Martella V, Pratelli A, Elia G, Decaro N, Tempesta M, Buonavoglia C. 2001. Isolation and genetic
33
34 14 characterization of two G3P5A[3] canine rotavirus strains in Italy. *J Virol Methods*
35
36 15 96(1):43-49.
37
38
39 16 Martella V, Colombrita D, Lorusso E, Draghin E, Fiorentini S, De Grazia S, Banyai K, Ciarlet M,
40
41 17 Caruso A, Buonavoglia C. 2008. Detection of a porcine-like rotavirus in a child with
42
43 18 enteritis in Italy. *J Clin Microbiol* 46(10):3501-3507.
44
45
46 19 Matthijssens J, Ciarlet M, Rahman M, Attoui H, Banyai K, Estes MK, Gentsch JR, Iturriza-
47
48 20 Gomara M, Kirkwood CD, Martella V, Mertens PP, Nakagomi O, Patton JT, Ruggeri FM,
49
50 21 Saif LJ, Santos N, Steyer A, Taniguchi K, Desselberger U, Van Ranst M. 2008.
51
52 22 Recommendations for the classification of group A rotaviruses using all 11 genomic RNA
53
54 23 segments. *Arch Virol* 153(8):1621-1629.
55
56
57 24 Nishikawa K, Hoshino Y, Taniguchi K, Green KY, Greenberg HB, Kapikian AZ, Chanock RM,
58
59 25 Gorziglia M. 1989. Rotavirus VP7 neutralization epitopes of serotype 3 strains. *Virology*
60
26 171(2):503-515.

- 1
2
3 1 Parashar UD, Gibson CJ, Bresse JS, Glass RI. 2006. Rotavirus and severe childhood diarrhea.
4
5 2 Emerg Infect Dis 12(2):304-306.
6
7
8 3 Phan TG, Trinh QD, Khamrin P, Kaneshi K, Ueda Y, Nakaya S, Nishimura S, Sugita K, Nishimura
9
10 4 T, Yamamoto A, Takanashi S, Yagyu F, Okitsu S, Ushijima H. 2007. Emergence of new
11
12 5 variant rotavirus G3 among infants and children with acute gastroenteritis in Japan during
13
14 6 2003-2004. Clin Lab 53(1-2):41-48.
15
16
17 7 Reidy N, O'Halloran F, Fanning S, Cryan B, O'Shea H. 2005. Emergence of G3 and G9 rotavirus
18
19 8 and increased incidence of mixed infections in the southern region of Ireland 2001-2004. J
20
21 9 Med Virol 77(4):571-578.
22
23
24 10 Sanchez-Fauquier A, Montero V, Moreno S, Sole M, Colomina J, Iturriza-Gòmara M, Revilla A,
25
26 11 Wilhelmi I, Gray J. 2006. Human rotavirus G9 and G3 as major cause of diarrhea in
27
28 12 hospitalized children, Spain. Emerg Infect Dis 12(10):1536-1541.
29
30
31 13 Santos N, Hoshino Y. 2005. Global distribution of rotavirus serotypes/genotypes and its implication
32
33 14 for the development and implementation of an effective rotavirus vaccine. Rev Med Virol
34
35 15 15(1):29-56.
36
37
38 16 Schumann T, Hotzel H, Otto P, Johne R. 2009. Evidence of interspecies transmission and
39
40 17 reassortment among avian group A rotaviruses. Virology 386(2):334-343.
41
42
43 18 Solberg OD, Hasing ME, Trueba G, Eisenberg JN. 2009. Characterization of novel VP7, VP4, and
44
45 19 VP6 genotypes of a previously untypeable group A rotavirus. Virology 385(1):58-67.
46
47
48 20 Suzuki Y, Gojobori T, Nakagomi O. 1998. Intragenic recombinations in rotaviruses. FEBS Lett
49
50 21 427(2):183-187.
51
52
53 22 Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of
54
55 23 progressive multiple sequence alignment through sequence weighting, position-specific gap
56
57 24 penalties and weight matrix choice. Nucleic Acids Res 22(22):4673-4680.
58
59
60 25 Trinh QD, Pham NT, Nguyen TA, Phan TG, Khamrin P, Yan H, Hoang PL, Maneekarn N, Li Y,
26
Kozlov V, Kozlov A, Okitsu S, Ushijima H. 2007. Amino acid substitutions in the VP7

- 1
2
3 1 protein of human rotavirus G3 isolated in China, Russia, Thailand, and Vietnam during
4
5 2 2001-2004. *J Med Virol* 79(10):1611-1616.
6
7
8 3 Trojnar E, Otto P, Johne R. 2009. The first complete genome sequence of a chicken group A
9
10 4 rotavirus indicates independent evolution of mammalian and avian strains. *Virology*
11
12 5 386(2):325-333.
13
14
15 6 Tsugawa T, Hoshino Y. 2008. Whole genome sequence and phylogenetic analyses reveal human
16
17 7 rotavirus G3P[3] strains Ro1845 and HCR3A are examples of direct virion transmission of
18
19 8 canine/feline rotaviruses to humans. *Virology* 380(2):344-353.
20
21
22 9 Wen L, Nakayama M, Yamanishi Y, Nishio O, Fang ZY, Nakagomi O, Araki K, Nishimura S,
23
24 10 Hasegawa A, Muller WE, Ushijima H. 1997. Genetic variation in the VP7 gene of human
25
26 11 rotavirus serotype 3 (G3 type) isolated in China and Japan. *Arch Virol* 142(7):1481-1489.
27
28
29 12 Yoshinaga M, Phan TG, Nguyen TA, Yan H, Yagyu F, Okitsu S, Muller WE, Ushijima H. 2006.
30
31 13 Changing distribution of group A rotavirus G-types and genetic analysis of G9 circulating in
32
33 14 Japan. *Arch Virol* 151(1):183-192.
34
35
36 15 Yu J, Langridge WH. 2001. A plant-based multicomponent vaccine protects mice from enteric
37
38 16 diseases. *Nat Biotechnol* 19(6):548-552.
39
40
41 17 Yuan L, Honma S, Ishida S, Yan XY, Kapikian AZ, Hoshino Y. 2004. Species-specific but not
42
43 18 genotype-specific primary and secondary isotype-specific NSP4 antibody responses in
44
45 19 gnotobiotic calves and piglets infected with homologous host bovine (NSP4[A]) or porcine
46
47 20 (NSP4[B]) rotavirus. *Virology* 330(1):92-104.
48
49
50 21 Zhou Y, Li L, Okitsu S, Maneekarn N, Ushijima H. 2003. Distribution of human rotaviruses,
51
52 22 especially G9 strains, in Japan from 1996 to 2000. *Microbiol Immunol* 47(8):591-599.
53
54
55 23
56
57 24
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1 **Figure Legends**

2

3 **Figure 1.** Percent temporal distribution of G1-G4 and G9 types of rotavirus strains circulating in
4 Palermo, Italy, from 1985 to 2005.

5

6 **Figure 2.** Phylogenetic analysis of nucleotide sequences of the VP7 gene of G3 strains. Italian
7 G3P[8] strains are highlighted in boxes, G3P[9] and G3P[3] are in bold. The origin of rotavirus
8 strains is indicated as follows: hu=human; ca=canine. The tree was generated based on the
9 neighbor-joining method using ClustalW program. Bootstrap values above 50%, estimated with
10 1,000 pseudoreplicate data sets, are indicated at each node.

11

12 **Figure 3.** Deduced amino acid sequences of the NSP4 protein of Italian G3P[8] strains collected
13 during the study period. The region involved in the binding to VP5* (aa 114-147) is indicated in
14 light grey. The VP6-binding region (aa 156-175) is marked in dark gray. The interspecies variable
15 domain (aa 131-141) is indicated by the thick dotted area. The sequence of the enterotoxigenic peptide
16 (aa 114-135) corresponds to the thin dotted area. Critical residues for antibodies recognition are
17 highlighted (▼). Conserved amino acid residues are indicated by dots. Wa/hu/G1P[8] is included as
18 the NSP4 genotype E1 rotavirus standard.

19