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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

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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

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)

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

¹Simona De Grazia, ²Vito Martella, ³Claudia Colomba, ⁴Antonio Cascio, ¹Serenella Arista,

^{1*}Giovanni M. Giammanco

Dipartimento di Scienze per la Promozione della Salute “G. D’Alessandro”, Sezione di

Microbiologia¹, Sezione di Malattie Infettive³, Università di Palermo;

Dipartimento di Sanità Pubblica e Zootecnica, Università di Bari²;

Clinica delle Malattie Infettive, Università di Messina⁴

Corresponding author: Dipartimento di Scienze per la Promozione della Salute “G. D’Alessandro”, via del Vespro 133, 90127, Palermo, Italia. Phone: +39 091 6553663; Fax: +39 091 6553676; e-mail address: g.m.giammanco@unipa.it (G.M. Giammanco).

Shortened title: Genotyping of Italian G3 rotaviruses

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Abstract

In recent years an apparent increase in the frequency of detection of G3P[8] rotaviruses has been observed worldwide. Similarly, in Italy G3P[8] strains have been detected sporadically and in a scattered fashion over 20 years, whereas in 2003 and 2005 G3P[8] rotavirus activity increased markedly. By analysis of the VP7, VP4, VP6 and NSP4 genes of a selection of G3P[8] rotaviruses detected between 1993 and 2005, a remarkable sequence conservation was observed in the VP7, VP4 and VP6 genes. By converse, after 2002 the Italian G3P[8] strains were found to possess unique mutations in significant regions of the NSP4 protein.

Keywords: rotavirus G3P[8], sequence analysis, NSP4, gastroenteritis, Italy

1 Introduction

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

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

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

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3 1 specific patterns of segregation may be revealed by phylogenetic analysis [Ciarlet et al., 2000]. By
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5 2 sequence analysis and PCR genotyping with specific primers, it has been shown that G1P[8],
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7 3 G3P[8], G4P[8] and G9P[8] strains possess a NSP4 E1 (B) genotype, while G2P[4] have NSP4 E2
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9 4 (A) genotype and G3P[9] viruses have either NSP4 E2 (A) or E3 (C) genotype [Estes and Kapikian,
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11 5 2007; Bányai et al., 2009].
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15 6 Rotavirus surveillance world-wide has shown in recent years changes in the distribution of
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17 7 rotavirus G types, with decrease of G1 strains and parallel increase of G3 strains (15.8%-63%) in
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19 8 Brazil, Japan, China, Russia, Ireland, Tunisia and Spain [Zhou et al., 2003; Fang et al., 2005; Lo et
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21 9 al., 2005; Reidy et al., 2005; Sanchez-Fauquier et al., 2006; Yoshinaga et al., 2006; Chouikha et al.,
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23 10 2007; Phan et al., 2007]. G3 rotaviruses infect almost all animal species and interspecies
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25 11 transmission and/or reassortment with animal rotaviruses accounts likely for the detection of human
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27 12 G3 strains with unusual genetic/antigenic features [Khamrin et al., 2006; De Grazia et al., 2007a;
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29 13 De Grazia et al., 2008; Tsugawa and Hoshino, 2008].
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34 14 In Italy, epidemiological surveillance for rotavirus enteritis in children has been carried out
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36 15 uninterruptedly since 1985 in Palermo. The possibility to analyse a collection of rotavirus strains
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38 16 spanning such a long time period has been useful for investigating the mechanisms of rotavirus
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40 17 evolution [Arista et al., 2006]. In this study, a selection of G3 strains identified over a long temporal
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42 18 range was analysed by comparing the genes coding for the outer capsid proteins VP7 and VP4, the
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44 19 inner capsid protein VP6 and the non-structural protein NSP4.
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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).

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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].

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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

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3 1 as previously described [Iturriza-Gòmara et al., 2004]. Amplicons were analyzed by electrophoresis
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5 2 with a 2% SeaKem LE (Cambrex Bio Science Rockland Inc., Rockland, ME) agarose gel in Tris-
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7 3 acetate-EDTA at 6 V/cm for 60 min. To determine the E and I genotypes, the NSP4 and VP6 genes
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9 4 were amplified by RT-PCR as described elsewhere [Lee et al., 2000; Iturriza-Gòmara et al., 2002].
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12 5 **Sequence and phylogenetic analyses.** Amplicons of the genes encoding VP4, VP6, VP7 and NSP4
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14 6 were directly sequenced with the specific primers. The amplicons were purified with GeneClean
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16 7 purification spin columns (Q-biogene, Cambridge, United Kingdom) prior to sequencing with the
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18 8 CEQ2000 Dye Terminator Cycle Sequencing Quick Start Kit (Beckman-Coulter, Fullerton, CA).
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20 9 All methods were carried out by following the manufacturers' instructions. Sequences were
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22 10 resolved with an automated sequencer (CEQ; Beckman-Coulter). Sequence alignment was
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24 11 performed with CLUSTAL W [Thompson et al., 1994] and phylogenetic analysis was carried out
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26 12 with the MEGA software, version 3.0 [Kumar et al., 2004], using the Kimura's two-parameter
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28 13 distance model of correction and the neighbor-joining method to construct the phylogenetic tree.
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30 14 The statistical significance of the inferred phylogenies was estimated by bootstrap analysis with
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32 15 1,000 pseudoreplicate data sets. The sequences were aligned with and compared to additional
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34 16 sequences obtained from online databases. Sequence alignment and phylogenetic analyses were
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36 17 carried out on partial VP7 (904 bp), VP4 (VP8*) (480 bp), VP6 (316 bp) and NSP4 (717 bp)
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38 18 fragments and on the deduced amino acids (aa).
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Results

In the surveillance period 1985-2006, among the 1591 rotavirus-positive samples obtained, 59 G3 rotaviruses (3.7%) were detected, with a high proportion (53/59=89.8%, or 3.3% of the rotavirus-positive samples) of G3P[8] strains. G3 strains were detected at low prevalence (0-6.1%) until 2002, whereas in 2003 and 2005 their prevalence reached 17% and 16.9%, respectively (Figure 1). Fifteen of the 53 strains exhibiting a G3P[8] type were selected in order to investigate their evolution (Table I). The VP7 serotype and the VP6 subgroup specificities of all of these strains were initially determined with MAbs, and all of the strains exhibited G3 serotype and subgroup II specificity. By sequence analyses of the VP6 gene, all the 15 G3P[8] strains were found to have a genotype I1, thus confirming the antigenic characterization with MAbs (data not shown). The Italian G3P[8] strains displayed >85% nt and 93.3-100% aa identity to reference I1 strains in the examined VP6 fragment. Nearly all of them tightly clustered with the reference I1 strain RMC437 (about 99% nt identity), but one strain, PA49/03, clustered with the I1 strain US0408 (99% nt identity).

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

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

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

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

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

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3 1 and reported to circulate at unusually high frequencies [Reidy et al., 2005; Phan et al., 2007; Trinh
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5 2 et al., 2007]. Whether the increased activity of such G3P[8] strains relies on intrinsic features of the
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8 3 VP7 and VP4, such as enhanced affinity for receptors or capsid stability, or on the properties of
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10 4 other viral proteins, is unclear.

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13 5 The NSP4 is a key protein involved in morphogenesis and pathogenesis of rotaviruses.
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15 6 NSP4 acts as intracellular receptor for single-shelled particles and mediates viral assembly into
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17 7 double shelled particles. NSP4 alone is able to induce diarrhea in experimental animals through a
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19 8 Ca⁺⁺-dependent signalling pathway and this activity has been mapped to the enterotoxigenic
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21 9 domain (aa 114 to 135). Antibodies against NSP4, or against the toxic peptide, are able to prevent
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23 10 watery diarrhea, although the precise “in vivo” mechanisms of protection are unknown [Ball et al.,
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25 11 2005; Estes and Kapikian, 2007] and the NSP4 is currently a target for vaccine development [Yu
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27 12 and Langridge, 2001; Choi et al., 2006]. The analysis of the NSP4 gene of the G3P[8] Italian
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29 13 isolates allowed their inclusion into genotype E1 (B), a finding that was not unexpected, due to the
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31 14 strength of the genetic linkage within each of the three human genogroups (Wa-, DS-1- and AU-1-
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33 15 like) [Bányai et al., 2009]. However, in the G3P[8] strains circulating in Palermo during 2003-2005,
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35 16 multiple NSP4 amino acid substitutions were detected with respect to strains circulating in the
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37 17 former years. These mutations affected the NSP4 region interacting with the VP4 (aa 114–147)
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39 18 [Hyser et al., 2008] that includes the interspecies variable domain (aa 131-141) [Yuan et al., 2004]
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41 19 and the sequence of the enterotoxic peptide (aa 114-135). Significant aa changes were observed also
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43 20 at residues considered to be critical for antibody binding to NSP4 epitopes (residues 111 and 137)
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45 21 [Hyser et al., 2008] and located within the VP6-binding region (residues 161 and 169) (Figure 3).
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47 22 Whether these punctuate mutations may account for increased virus fitness, influenced by the
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49 23 evolution of the NSP4-VP5* and/or NSP4-VP6 binding domains, or the appearance of NSP4
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51 24 variants may facilitate the escape from acquired immunity is an intriguing possibility and surely
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53 25 deserves attention. The NSP4 aa pattern encountered in most of the 2005 Italian strains is not
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55 26 completely novel, since it has already been described in a G3P[8] strain collected in Leeds (UK) in
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1999 [Iturriza-Gòmara et al., 2003]. In addition, Brazilian G1P[8] and G9P[8] strains with NSP4 patterns resembling the 2003 and 2005 G3P[8] NSP4 patterns have been detected in 2001, 2003 and 2004 [Araujo et al., 2007; de Moraes Tavares et al., 2008].

In conclusion, analysis of G3P[8] strains detected in Palermo, Italy, over nearly 20-years of uninterrupted rotavirus surveillance revealed that these rotavirus strains tended to be highly conserved. The low heterogeneity of G3P[8] rotaviruses could be accounted for by the observed low prevalence in the infantile population, preventing the onset of immune-pressure mechanisms. The apparent increase in G3P[8] activity observed in Italy and in other countries worldwide remains unclear. However, in this study some aa mutations in significant regions of the NSP4 protein allowed to differentiate the old strains from the recent G3P[8] viruses, suggesting a possible correlation with the G3P[8] peaks observed in 2003 and 2005. These findings may provide clues for understanding of the mechanisms of evolution of rotavirus, which could be addressed in more extensive investigations based on national and international surveillance programmes of rotavirus.

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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%).

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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].

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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.

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