Data mining from a 27-years rotavirus surveillance in Palermo, Italy

Simona De Grazia a,*, Floriana Bonura a, Claudia Colomba a, Antonio Cascio b, Francesca Di Bernardo c, Antonina Collura c, Diane M. Terranova c, Vito Martella d, Giovanni M. Giammanco a

a Dipartimento di Scienze per la Promozione della Salute e Materno infantile “G. D’Alessandro”, Universita’ di Palermo, Palermo, Italy
b Dipartimento di Patologia Umana, Universita’ di Messina, Messina, Italy
c Unità Operativa di Microbiologia e Virologia, Ospedale Civico e Di Cristina, ARNAS, Palermo, Italy
d Dipartimento di Sanità Pubblica e Zootecnia, Universita’ Aldo Moro di Bari, Valenzano, Italy

1. Introduction

Group A rotaviruses, family Reoviridae, are the most important agents of acute gastroenteritis in young children and livestock animals worldwide (Estes and Kapikian, 2007). Rotavirus virions have a triple layered capsid with a genome of 11 segments of double stranded RNA. Group A rotaviruses (RVA) are classified on the basis of antigenic and genetic diversity of the two outer capsid proteins VP7 and VP4, both eliciting neutralising antibodies, in G and P-types. Actually, according to established nucleotide percent cut-off values, have been identify at least 27 G and 37 P types, respectively, with at least 73 G/P combinations being described thus far (Matthijnssens et al., 2011a; Trojnar et al., 2013). The majority of the medically important human rotaviruses belong to five G/P combinations, i.e. G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] (Estes and Kapikian, 2007). G1P[8] are universally acknowledged as the most prevalent and ubiquitous RVAs (Banyai et al., 2012), G2P[4], G3P[8], G4P[8] and G9P[8] strains are common but their incidence varies regionally and temporally (Banyai et al., 2012). Other G/P combinations sporadically appear to acquire relevance in some geographical settings and populations. Rearrangement, reassembly, interspecies transmission and positive accumulation of point mutations represent the main forces driving the evolution of rotaviruses and are responsible for the production of an heterogeneous population of viruses in continuous evolution (Papp et al., 2013).

In Palermo, Sicily, South of Italy, a documented uninterrupted RVA surveillance has been conducted since 1985 (Arista et al., 1986, 1990, 1997, 2003, 2004, 2005a,b, 2006; Colomba et al., 2006; De Grazia et al., 2007a,b, 2008, 2009, 2010, 2011; Giammanco et al., 2014). This archival collection, spanning more than 25 years, offers a unique instrument to evaluate the patterns of variation of RVA infections over the years and to investigate the genetic diversity of the strains circulating in a settled population. Other archival collections of a similar extent have been studied in Argentina and Russia but the epidemiological and/or sequence information extracted from these biological archives was incomplete and/or partial (Barril et al., 2013; Novikova et al., 2012). Sicily, the largest island in the Mediterranean sea, for its proximity to the African shores has become in the last decade one of the principal gateways to Europe for illegal immigration, and thus represents a privileged position to monitor the introduction and spread of novel RVA strains through population movements.

* Corresponding author. Address: Dipartimento di Scienze per la Promozione della Salute e Materno infantile “G. D’Alessandro”; Universita’ di Palermo, Via del Vespro 133, 90127 PA, Italy. Tel./fax: +39 091 6553663.
E-mail address: simona.degrazia@unipa.it (S. De Grazia).
Two rotavirus vaccines have been licensed in 2006 and are now available in 18 countries. Since their introduction, an effectiveness of 85–90% has been estimated (Vesikari, 2012). Starting from May 2012, Sicily introduced universal rotavirus vaccination in the regional vaccination schedule. The surveillance data on the rotavirus prevalence and types circulating over the last 27 years will offer a valuable background for assessing the benefits of the newly introduced RVA vaccine program on human health. They will also contribute to understand whether the RVA vaccine can alter the epidemiology of RVAs or select the onset of new RVA strains in a population.

In this study, the 27-years RVA surveillance data are summarised and illustrated in detail, providing an in-depth investigation of the temporal pattern of RVAs variation in the pre-vaccine era.

2. Methods

2.1. Study population

RVA surveillance in Palermo, Italy, started on January 1985. From 1985 to August 2012, a total of 6522 stool samples were collected from children under 5 years of age hospitalised with acute gastroenteritis at the “G. Di Cristina” Children’s Hospital. Acute gastroenteritis was defined by at least 3 watery stools with or without bouts of vomiting in 24 h and of less than 7 days of duration, with no identifiable symptoms other than those related to infective gastroenteritis. Stool samples were collected within 12 h after admission to the hospital to avoid inclusion of nosocomial cases and stored at −20 or −80 °C until processing. Since the RVA season in Palermo is generally delayed with respect to the usual winter season and peaks of RVA circulation occur from January to April, the term “year” was used as synonym of “season” in this study.

2.2. G and P genotyping

Over the study period, G typing was performed by enzyme immunoassay until 2005, using type G1-, 2-, 3-, 4-, 6-, and 9-specific neutralising monoclonal antibodies (MAb) reactive with the viral protein VP7 (Arista et al., 1990). After 1999, MAb typing was performed in parallel with RT-PCR genotyping of VP7 gene with primers specific for G1–G4, G6 and G9. From 2008 also specific primers for G8–G12 genotypes have been included. To confirm serotyping results, RT-PCR genotyping was performed retrospectively for selected samples and became the sole G typing procedure starting from 2002. The primer sets were updated over the time following the literature (Arista et al., 1990; Gouvea et al., 1990; Iturriza-Gomara et al., 2004). Likewise, starting from 1999 the P genotyping was carried out routinely and performed retrospectively for selected samples and became the sole G typing procedure starting from 2002. The primer sets were updated over the time following the literature (Arista et al., 1990; Gouvea et al., 1990; Iturriza-Gomara et al., 2004). Likewise, starting from 1999 the P genotyping was carried out routinely and performed retrospectively for selected samples and became the sole G typing procedure starting from 2002. The primer sets were updated over the time following the literature (Arista et al., 1990; Gouvea et al., 1990; Iturriza-Gomara et al., 2004).

2.3. Sequence and phylogenetic analyses

Strains representative of the whole study period were selected for sequence analysis for each genotype. The nearly full-length VP7 gene and the VP8* portion of the VP4 gene were amplified with consensus primers and the sequences were determined by direct sequencing. Sequence alignment was performed with CLUSTAL W (Thompson et al., 1994). Phylogenetic analysis was carried out using the MEGA software version 5.0 (Tamura et al., 2011), using the Kimura 2-parameter model as a method of substitution and the neighbour-joining method to construct phylogenetic trees from partial sequences of VP7 and VP4. The statistical significance of the phylogenies inferred was estimated by bootstrap analysis with 1000 pseudoreplicate data sets.

3. Results

During a 27-years surveillance for rotavirus infections in children hospitalised with diarrhoea to the “G. Di Cristina” Children Hospital of Palermo, RVA infection was detected in 32.8% (2138) of 6522 samples tested. RVAs yearly prevalence rates ranged from 11% in 1987 to 52% in 2005. RVA seasonality in Palermo showed peaks of activity from the end of winter to mid-spring (Fig. 1). A G-type was determined for 2018 (94.4%) out of 2138 RVA strains. Strains with G1, G2, G3 and G4 specificities accounted for more than 90% of infections before 1999, when RVAs of the G9P[8] type emerged locally. The analysis of the temporal distribution of the most common RVA G/P combinations over the study period revealed that RVA strains belonging to the G1P[8] type were the most common and they were constantly detected in the last 27-year surveillance period with a relative prevalence varying from a maximum of 95.8% in 1989 to a minimum of 6.4% in 2006 (Fig. 2). On the contrary, the other four main rotavirus G/P combinations were not continuously observed through the years. G2P[4] exhibited marked fluctuations in their patterns of circulation. A significant activity of G2P[4] strains was observed in 1996 and 1997 (50% and 38%, respectively), 2003 (22.2%), 2007 (14.1%), 2011 (17.2%) and 2012 (13.3%). G3P[8] rotavirus circulation was limited and accounted generally for less than 6.3% of the RVA strains, but in 2003 and 2005 when they were involved in 16.7% and 17.1% of the gastroenteritis episodes, respectively. G4P[8] epidemics occurred in three periods: 1990–1993, 1999–2001 and 2003. G9P[8] RVAs emerged in Palermo in 1999, when they represented 32.4% of the strains detected, and therefore their circulation fluctuated, almost disappearing and therefore re-emerging (in 2005, 2006 and 2008). However, in the years 1999–2010, G9P[8] represented the second most common strain after G1P[8] (Fig. 2). During the surveillance period unusual, animal-like, human strains were occasionally detected. Three human/animal reassortant G3P[9] strains were detected in 1994, 1996 and again in 2011 (De Grazia et al., 2008, 2010). A G3P[3] canine-like RVA was isolated in 1997 (De Grazia et al., 2007b), while G6 strains were sporadically isolated starting from the late 1980s in association with either P[9] or P[14] VP4 specificity (De Grazia et al., 2011). A G10 strain and three G12 rotaviruses were detected in 2011 and 2012, respectively.

3.1. Sequence analysis of the VP7 gene of G1 RVA strains

Sequence and phylogenetic analyses of the various VP7 and VP4 genotypes revealed a high heterogeneity, with different intra-genotype lineages and sublineages. Upon molecular analysis of the VP7 gene, significant sequence variation was observed within the G1 strains, clustering into three distinct genetic lineages, i.e., I, II and V. Moreover, three and four sublineages could be defined within lineages I (Ia-Ic) and II (Ila-d), respectively. Sublineage Ia included strains detected over twelve years, from 1986 to 1997; sublineage Ib included only two 2001 strains; sublineage Ic included 28 strains spanning sixteen years, from 1996 to 2012. Sublineage IIa included eleven strains spanning from 1989 to 1996; sublineage IIb included two strains of 1994 and 2005; sublineage IIc included three strains of 2003 and 2004, and one of 2012; sublineage IIId included nine strains collected from 1995 to 2002. Three strains of 1989 and 1990 segregated into lineage V. No G1 RVA strains were identified in lineage III, where reference strain Wa and vaccine strain RotaTeq Wt79-9 segregated (Fig. 3a).
3.2. Sequence analysis of the VP7 gene of G2, G3, G4 and G9 RVA strains

Similarly to G1 RVAs, the G2 strains could also be differentiated into lineages and sublineages based on sequence analysis of the VP7 gene. The older G2P[4] strains, detected in the 1990s, were assigned to lineage II, while the 2004–2006 and the 2007–2011 RVAs clustered into sublineage IVa-1 and sublineage IVa-3, respectively (Fig. 3b). Among the G3 strains a lower heterogeneity was observed. The G3 RVAs associated with a P[8] genotype segregated into sublineage 3d, while animal–like G3P[3] and G3P[9] strains, which were detected only sporadically over the time, segregated into lineage 1 and into sublineage 3c, respectively (Fig. 3c). The G4 RVAs segregated into three well-defined sublineages (Ia-c). Nine strains, collected from 1990 to 1994, clustered into sublineage Ia, a single strain of 1995 was in sublineage Ib, and all the G4 strains collected from 1999 onward (1999, 2000, 2003 and 2012) segregated into sublineage Ic (Fig. 3d). Since the first identification of G9 strains in Italy in 1999 (Arista et al., 2004), a homogeneous population of G9 RVA strains was detected in Palermo, with all the G9 VP7 sequences being included into lineage III (Fig. 3e).

3.3. Sequence analysis of the VP7 gene of G6, G10 and G12 RVA strains

Seven G6 RVAs were identified in Palermo in a scattered fashion and found to be associated with either P[9] or P[14] types: two G6P[9] were detected in 1987, 1993 and two in 2003; three G6P[14] were detected in 1988, 1989 and 2002 (De Grazia et al., 2011). Upon VP7 analysis the G6 strains were grouped into different sublineages with a P genotype-linked pattern. The G6P[9] rotaviruses clustered in G6 sub-lineages Ia, Ic and Id, while the G6P[14] strains segregated into sublineages Ila and Ild (Fig. 3f). A G10 RVA was identified in 2011 and its VP7 segregated along with a novel G10P[14] rotavirus strain circulating in the Northern Territory of Australia (Fig. 3g). The G12 strains were detected in Sicily only in 2012 and they all clustered into VP7 lineage III (Fig. 3h).

3.4. Variation of the VP7 epitopes

Amino acid (aa) substitutions with respect to Rotarix and RotaTeq vaccine strains were searched in the relevant VP7 antigenic regions of the common G types analysed in the study (Zeller et al., 2012). All the G1P[8] strains detected in this study differed from the RotaTeq vaccine strain WI79-9 at positions 97 and 147 in the 7-1a and 7-2 neutralising domains, respectively. Four additional aa substitutions in the VP7 antigenic epitopes with respect to the two vaccine strains WI79-9 and A41CB052A (Rotarix) were identified in subsets of G1 strains detected in Sicily. In particular, lineage G1-I strains shared three aa changes in domain 7-1a (residues 123 and 291) and 7-2 (residue 217), the first substitution being also shared by sublineage IIa G1 strains. Some Ic G1 strains also showed aa substitutions at positions 96 and 100 (domain 7-1a), 212 (domain 7-1b) and 190 (domain 7-2). In the Sicilian G2P[4] strains, the various lineages named according to Doan’s classification (Doan et al., 2012) showed different patterns of aa mutations with respect to the RotaTeq vaccine strain.

Fig. 3. Phylogenetic analysis of partial VP7 nucleotide sequences of genotype G1 (a), G2 (b), G3 (c), G4 (d), G9 (e), G6 (f), G10 (g) and G12 (h) strains. The strains from this study are indicated in bold. Some of the strains are replaced by triangles, the height of the triangle represents the number of sequences included. Rotavirus nomenclature has been used according to the Rotavirus Classification Working Group (RCWG) (Matthijnssens et al., 2011a). Phylogenetic trees were constructed using the neighbor-joining method with the kimura-2-parameter. Bootstrap values (1000 replicates) above 70% are shown.

SC2-9, progressively involving up to four aa positions: 87 and 96 (domain 7-1a), 213 and 242 (domain 7-1b). The Sicilian G3P[8] VP7 differed from the RotaTeq vaccine strain WI78-8 at three positions in domain 7-1b (positions 212, 238 and 242). The G4P[8] Sicilian strains showed a peculiar aa change at position 212 in domain 7-1b with respect to the RotaTeq vaccine strain.
BrB-9, with two additional substitutions appearing in the strains circulating in 1990–1994 in domain 7-1a (position 125) and 7-1b (position 201). These additional mutations were not observed in the strains circulating from 1999 onward (1999–2003 and 2012), but a new mutation appeared at position 146 (domain 7-2) (Fig. 4a).

3.5. Sequence analysis of the VP8* portion of the VP4 gene

Sequencing of the VP8* portion of the VP4 gene revealed the presence of two discrete lineages among P[8]-type strains: P[8]-I and -III. A temporal pattern was observed, with the strains detected from 1989 to 2002 clustering into lineage P[8]-I, while the most recent strains, and some strains identified in 1993, clustered into the lineage P[8]-III (Fig. 5a).

Likewise, the P[4] VP8* sequences of G2 strains detected in the 1990s clustered into lineage P[4]-II, while the 2004–2011 P[4] strains clustered into two distinct P[4]-IV sub-lineages. The RVAs of 2004, 2006 and 2008 segregated into sub-lineage IVa, while the majority of the most recent strains (2007, 2008, 2010 and 2011) fell into the sub-lineage IVb (Fig. 5b) (Giammanco et al., 2014).

In both P[9] and P[14] Italian strains three separate lineages were detected (Fig. 5c and d).

In the inferred aa sequences, the Italian P[8] strains differed for peculiar aa changes in the epitopes 8-1 and 8-3 of VP8* from the Rotarix and RotaTeq vaccine strains. The P[8]-III strains displayed more aa changes from both vaccine strains compared to the older P[8]-I RVAs. In particular, the P[8]-I strains completely matched the Rotarix-A41CB052A strain in the antigenic epitopes but differed from the vaccine strain Rotateq WI79-4 strain at aa 190 (N–S) and at aa 196 (D–N). By converse, the P[8]-III strains differed in four aa substitutions from the P[8] vaccine strains, with three aa changes in epitope 8-1 and a further change in epitope 8-3. In detail, all the P[8]-III strains displayed two mutations at positions 146 (S–G) and 196 (N/D–G) of epitope 8-1. An additional mutation at aa 195 of epitope 8-1 (N–D) was found in the majority of the P[8]-III strains detected from 2005 onward. In the epitope 8-3, 43 out of 103 P[8]-III strains, mostly composed of G1 strains detected until 2009 and of G9 strains circulating in the 1999–2001 time span, had the mutation N–D at position 113 (Fig. 4b).

4. Discussion

In the 27-years surveillance, the overall prevalence of RVA infection (32.7%) confirmed its role as a major agent of enteritis in children. In Sicily, RVA infections occurred throughout the year with wide fluctuations and increased circulation from October to May. Therefore, RVA activity in Sicily seems to peak with a 1–2 months delay with respect to Northern and Central Italy (Ruggeri et al., 2011). In Europe, rotavirus infection peaks occur usually in the winter months but the epidemic waves seem to start in the South-West (Iberian peninsula) and subsequently spread to the North-East (Iturriza-Gomara et al., 2011). It is possible that in Italy RVA infection wave follows a North to South direction.

The data presented in this study gave us a comprehensive picture of the genetic diversity of the RVAs circulating in Sicily over the last three decades. Although the vast majority of the RVA strains detected in Sicily displayed “common” RVA G/P genotype combinations, unusual RVA types (G3P[9], G3P[3], G6P[9] and G6P[14]) were occasionally detected. In a recent systematic review of global rotavirus diversity in humans, G1, G3, G4 and G9P[8] and G2P[4], have been recognised as the major rotavirus circulating...
Fig. 5. Phylogenetic analysis of the VP8* portion of VP4 nucleotide sequences of P[8] (a), P[4] (b), P[9] (c) and P[14] (d) strains. The strains from this study are indicated in bold. Some of the strains are replaced by triangles, the height of the triangle represents the number of sequences included. Rotavirus nomenclature has been used according to the Rotavirus Classification Working Group (RCWG) (Matthijnssens et al., 2011a). Phylogenetic trees were constructed using the neighbor-joining method with the kimura-2-parameter. Bootstrap values (1000 replicates) above 70% are shown.

genotypes, accounting for more than 88% of all strains identified worldwide. Changes in the relative frequencies were observed over time, indicating a decline of G1P[8] RVAs in the last decade (Banyai et al., 2012). However, in Palermo G1P[8] strains were constantly detected during 27 years and they were the most common strain in most years, followed by G9P[8] (since 1999) and G4P[8] RVAs. These findings are in agreement with the epidemiological data described in other Italian regions, in many European countries,
and in the majority of the developed countries (de Rougemont et al., 2011; Iturriza-Gomara et al., 2004, 2011; Santos and Hoshino, 2005; Zuccotti et al., 2010).

In a previous study, we hypothesized that the continuous circulation and predominance of G1P[8] strains in Sicily was warranted by their high genetic/antigenic heterogeneity, with local circulation, co-circulation, emergence and re-emergence of different lineages and sublineages (Arista et al., 2006). In addition to the previous observations, from 2007 to 2012 the pre-dominance of G1 RVAs in Palermo was sustained by sub lineage lc strains. This sublineage re-emerged locally in 2007 after previous circulation in 1996, 1997, 2004 and 2005. Interestingly, in 2009 a mutation occurred in these strains at residue 212 (V–T) in the 7-bp epitope. This residue has also been found to vary in G3P[8] strains detected after vaccine introduction (Zeller et al., 2012). In our study this substitution appeared independently of selective pressure induced by vaccines. Whether persistence and dominance of G1 RVAs may be associated to alternate circulation of different variants is not still clear (Barril et al., 2013).

G2P[4] and G4P[8] strains showed a fluctuating circulation pattern, cyclically reaching high prevalence rates in Palermo (Arista et al., 2005a,b). The G2P[4] waves in local population were caused by different G2 VP7 lineages, with the replacement of the ancestral lineage II by lineage IVA-1 in 2004, followed by the onset and circulation of lineage IVA-3 from 2007 to 2011 (Giammanco et al., 2014). The replacement of G2 VP7 lineages was not only a local phenomenon as these changes occurred globally with a similar chronology (Doan et al., 2012). Lineage II G2 RVAs were predominant in the 1990s, while RVAs of lineage IV dominated globally in the 2000s, with two distinct sub-lineages, IVA-1 and IVA-3, emerging consecutively. Full genome analysis of G2P[4] strains representative of the three VP7-lineages has revealed that the emergence of G2 variants was also combined with major shifts in the genome composition, with acquisition of genome segments from rotavirus of ruminants (Giammanco et al., 2014).

G3P[8] appeared and disappeared over the years and never reached relevant prevalence rates in Palermo. Also, G3P[8] RVAs displayed a remarkable conservation in the VP7 gene (De Grazia et al., 2009). In some Western Pacific Asian countries, G3 RVAs have been recognised as the dominant type in recent years (Banyai et al., 2012; Thongprachum et al., 2013). This increased circulation of G3 RVAs was related to the emergence of a new G3 variant, classified as VP7 lineage 3d, and characterised by a peculiar pattern of aa substitutions in the antigenic epitopes (Thongprachum et al., 2013). All the Italian G3P[8] strains, detected from 1993 to 2012, were characterised as lineage 3d and were closely related to this novel G3 variant.

Since their onset in Palermo in 1999, G9P[8] strains were constantly identified until 2010. From 1999 to 2010, G9 strains exhibited high prevalence rates in Palermo, with a peak in 2006 when they were the predominant strains. G9 RVAs were first detected in humans in the 1980s and, since then, they spread quickly in different countries worldwide acquiring in several settings a predominant epidemiological role (Armagh et al., 2010; Cunliffe et al., 1998; Nakagomi et al., 1990). In Palermo, we observed two waves of G9 RVAs, in 1999–2001 and in 2005–2009, sustained by two distinct VP7 sublineages. Unusual G/P combinations were occasionally detected in Palermo. G3 strains in association with either P[3] or P[9] and G6 strains with either P[9] or P[14] specificity were found only sporadically (De Grazia et al., 2007b, 2008, 2010, 2011). Human G3P[3] and G3P[9] viruses are believed to be transmitted to humans directly from cats and/or dogs, respectively (Martella et al., 2011; Matthijnssens et al., 2011b). Interestingly, although the feline-like multi-reassortant G3P[9] strains in Sicily were detected over a wide temporal span (1994, 1996 and 2011), they retained a stable genetic constellation. Likewise, the unusual G6P[9] and G6P[14] RVAs were detected sporadically in 1987–88, and 2003 (De Grazia et al., 2011). Full-length genome sequencing of G6 strains has revealed a close genetic relatedness between human G6P[14] and animal G6P[14] and G8P[14] viruses, reinforcing the hypothesis that animal viruses impact heavily on the evolution of RVAs (Banyai et al., 2010; De Grazia et al., 2011; Martella et al., 2011; Matthijnssens et al., 2009). The emerging G10 and G12 RVAs were detected for the first time in our geographic region in 2011 and 2012, respectively.

5. Conclusion

A number of surveillance studies have investigated the diversity of RVA genotypes, suggesting that the predominant RVA types change seasonally (Banyai et al., 2012). During our 27-years long study marked fluctuations in the circulation of non-G1 RVAs were documented, while G1P[8] RVAs remained the prevalent strains over most of the seasons. In Palermo, a portrait of the evolutionary dynamics of medically important RVAs in a geographically defined population and in a very long span of time was obtained. Intrageneric diversity was observed, with the onset of novel RVA variants displaying punctate mutations in the VP7 and VP4 aa sequence. Persistence and dominance of G1 RVAs may be associated to alternate circulation of different VP7 lineages and sublineages emerging and re-emerging consecutively and in different combinations with VP8’ lineages. The waves of non-G1 RVAs increased circulation were also generally associated to novel VP7 lineages but for reasons that still need to be clarified the frequency of their emergence and spread seems to be much more limited than for G1 strains. RVAs are subjected to a continual transformation involving not only accumulation of point mutations, but also reassortment events with human and animal viruses which have been shown to trigger the onset of novel strains (Martella et al., 2011). Large-scale surveillance studies and the accumulation of whole genome sequencing data will provide new insights into the mechanisms driving the evolution of RVAs (Matthijnssens et al., 2008, 2011a,b). This work allows documenting rotavirus pre-vaccine era in Sicily and will provide a valuable baseline information for RVA surveillance activities after the introduction of universal vaccination in the region.

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