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FULL PAPER

Rotavirus molecular epidemiology in hospitalized patients, Northern Italy, 2015-2018

Francesca Rovida[1], Edoardo Vecchio Nepita[2], Federica Giardina[1,2], Antonio Piralla[1], Giulia Campanini[1], Fausto Baldanti[1,3]

[1]*Molecular Virology Unit, Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy;*
[2]*Specialization School in Clinical Microbiology and Virology, Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Italy;*
[3]*Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Italy*

SUMMARY

About 15,000 hospitalizations due to group A Rotavirus gastroenteritis (RVA) are recorded each year in Italy. In the present study, we report the seasonal distribution and molecular characterization of RVA in pediatric and adult hospitalized patients in the period September 2015-April 2018 in Pavia province, Lombardy Region. During the study period, stool samples of 1450 patients with acute gastroenteritis were analyzed and 122 were RVA positive, the majority belonging to pediatric patients (94.0%) while only a minority of patients (6.0%) were adults. G3P[8], G1P[8], G9P[8] and G2P[4] were the most detected RVA strains, with a prevalence of 82.4%. However, a variety of RVA strains circulated in Northern Italy in hospitalized patients over a period of three years, emphasizing distinct patterns of distribution in different age groups and between years.

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INTRODUCTION

Group A Rotavirus (RVA) is a major cause of acute gastroenteritis in young children worldwide, leading to approximately 215,000 death in children <5 years of age every year (Tate *et al*., 2016). Each year, RVAs are a ssociated with approximately 111 million episodes of acute gastroenteritis requiring home care, 25 million clinic visits and 2 million hospitalizations, representing a major economic burden for health care systems and families in both the US and European countries (Parashar *et al*., 2006; Soriano-Gabarrò *et al*., 2006; Forster *et al*., 2009). The majority of children hospitalized with RVA infection presented with diarrhea, vomiting, and fever. Virtually every child around the globe experiences RVA diarrhea in the first 3-5 years of life (WHO, 2007). RVA infection is less frequent in adults; nevertheless, chronic infection has been described in immunocompromised patients (Peigue-Lafueille *et al*., 1991). Rotaviruses (RV) are non-enveloped double-stranded RNA (dsRNA) viruses with a complex architecture of three concentric capsids that surround a genome of 11 segments of dsRNA. The RNA segments encode six structural viral proteins (VP1, VP2, VP3, VP4, VP6, and VP7) and six non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5, and NSP6) (Estes and Greenberg, 2013). RVs are classified into different species (A-J) on the basis of different genome profiles and antigenic differences of VP6 (Matthijnssens *et al*., 2012; Mihalov-Kovács *et al*., 2015; Bànyai *et al.,* 2017). RV belonging to genogroups A, B, C, and H are associated with human infections (Matthijnssens *et al*., 2012; Mihalov-Kovács *et al*., 2015) and the viruses belonging to genogroup A are the most frequently implicated in human gastrointestinal infections (Matthijnssens *et al*., 2011). RVAs are further classified into different genotypes, based on genetic differences in RNA segments 7 and 4, encoding respectively for VP7 and VP4 proteins, which form the basis of the dual nomenclature system used for RVA strains, whereby glycoprotein (G or VP7) and protease-cleaved protein (P or VP4) subtype are differentiated (Estes and Greenberg, 2013). Geographical differences in RVA strains distribution have been observed, with more strains causing RVA disease in children in developing countries than in developed countries (Gentsch *et al*., 2005; Nakagomi and Nakagomi, 1991). Although several G and P genotype combinations have been described, six strains of RVA: G1P[8], G2P[4], G3P[8], G4P[8] G9P[8] and G12P[8] are responsible for the majority of RVA infections in children (Dóró *et al*., 2014; Iturriza-Gòmara *et al*., 2011). With the advent of molecular techniques such as Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), classification into G and P type on the basis of genes encoding VP7 and VP4 proteins respectively has become a widely accepted standard technique (Jayaram *et al*., 2004). Virus evolution is driven by the accumulation of point mutations and genome reassortment, which can occur after dual infection of individual cells by different RVA strains, leading to viral progeny with a combination of the parental genomes (Crawford *et al*., 2017).

In the present study, we report the molecular characteriza-

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Corresponding author:
Fausto Baldanti
E-mail: f.baldanti@smatteo.pv.it;
fausto.baldanti@unipv.it

tion of RVA in hospitalized patients in the period September 2015-April 2018 in Pavia, Lombardy Region, Northern Italy.

MATERIAL AND METHODS

Patients and Samples collection

In the period September 2015-April 2018 stool samples of 1450 patients with gastroenteritis hospitalized at the Fondazione IRCCS Policlinico San Matteo of Pavia (a teaching and university hospital with 36,500 admissions, 2,100,000 outpatient visits and 99,000 emergency consultations per year), Italy, were analyzed to evaluate a potential RVA infection. The stool samples were tested for the presence of RVA with the routine immunochromatographic assay (RIDA®QUICK Rotavirus, R-Biopharm AG, Darmstadt, Germany). The positive samples (122/1450, 8.4%) were stored at -80°C and retrospectively analyzed with RVA molecular assays for strain characterization.

Viral nucleic acid extraction and RVA genotyping

Viral nucleic acids were extracted from 400 µL of 10% stool suspension, using the automated extractor QIAsimphony®SP with DSP Virus/Pathogens MiDi Kit, version 1 (QIAGEN, Hilden, Germany) coextracting DNA and RNA in a final elution volume of 55 µL, according to manufacturers' instructions. The identification of G and P RVA genotype was performed by specific Reverse Transcriptase Polymerase Chain Reactions (RT-PCRs), amplifying 5 µL of extracted nucleic acid for each amplification, using the Ag-Path-ID 1-step RT-PCR kit (Life Technologies, Austin, Texas, USA) according to the manufacturer's instructions. As described in detail in *Table 1*, VP7F and VP7R consensus primers (Iturriza-Gòmara *et al*., 2001) were used for identification of G type and Con2 and Con3 consensus primers (Gentsch *et al*., 1992) were used for P typing. The RT-PCR products were detected by electrophoresis on 3% agarose gel.

Sequencing

Purified RT-PCR products were sequenced using the Big-Dye Terminator Cycle-Sequencing kit (Applied Biosystem, Foster City, CA, USA) with an ABI Prism 3130xl Genetic Analyzer (Applied Biosystem). Sequences were assembled using the Sequencer software, version 4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). The genotype was determined by comparing sequence products to GeneBank reference strains through the Basic Local Alignment Search Tool (BLAST).

Ethics statement

This study was performed according to guidelines of the Institutional Review Board of the Fondazione IRCCS Policlinico San Matteo on the use of biological specimens for scientific purposes in keeping with Italian law (Art. 13 Decree Law 196/2003).

RESULTS

Patients and samples

Overall, 1450 stool samples from 1450 patients with gastrointestinal syndromes were analyzed for RVA with

Table 1 - *Molecular parameters used for RVA genotyping.*

Primer	*Gene target*	*Thermal profile*	*Cycle no.*	*Oligonucleotide sequence (5' to 3')*	*Size*	*References*
VP7 F	VP7	45°C/10' 95°C/10'	1	ATG TAT GGT ATT GAA TAT ACC AC	881	Iturriza-Gòmara, *et al*., 2001
VP7-R	VP7	94°C/1' 50°C/2' 72°C/2'	25	AAC TTG CCA TTT TTT CC		
Con-3	VP4	45°C/10' 95°C/10'	1	TGG CTT CGC CAT TTT ATA GAC A	876	Gentsch *et al*., 1992
Con-2	VP4	95°C/1' 50°C/1' 72°C/1'	35	ATT TCG GAC CAT TTA TAA CC		

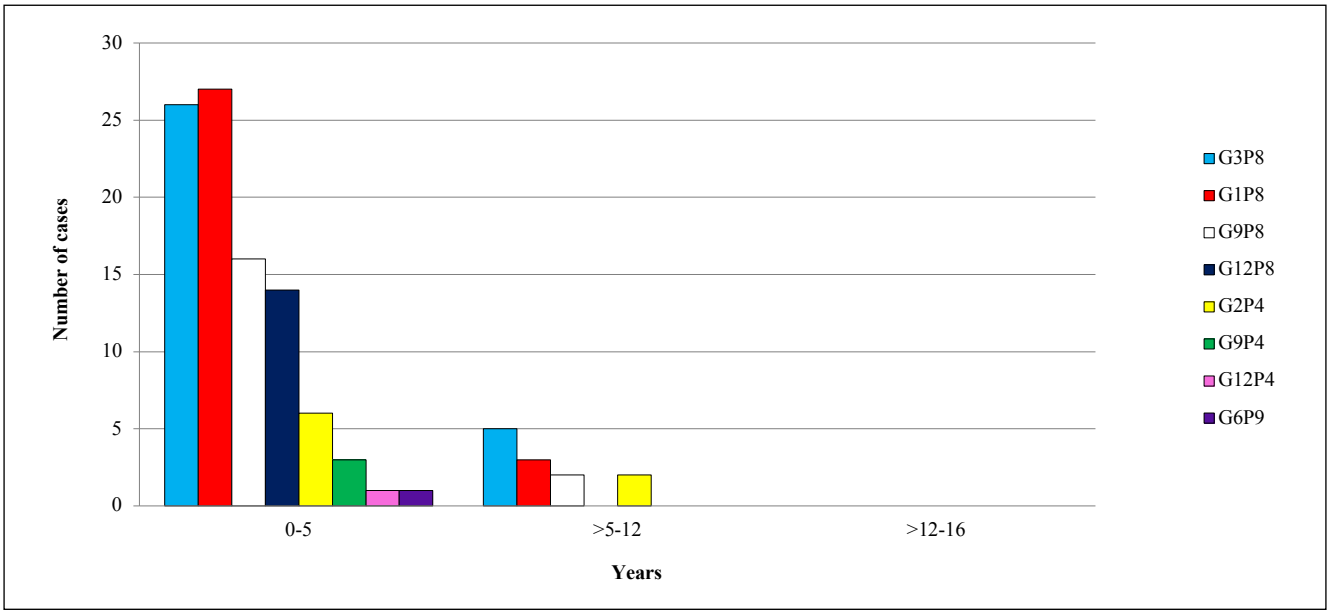

Figure 1 - *Distribution of RVA genotypes in hospitalized pediatric patients, according to age groups.*

the specific immunochromatographic assay and 122 (122/1450, 8.4%) were positive. 115 (115/122, 94%) of patients with RVA infection were pediatric and 7 (7/122, 6%) were adults. The age of pediatric patients ranged from less than 1 month to 16 years (median, 29 months), whereas the age of adult patients ranged from 17 to 88 years (median, 45 years). The positive samples were submitted to further molecular analysis and RVA RNA was detected in 111 samples out of 122 (90%); genotyping was obtained in 108 of the 111 (97.3%) positive samples. The vast majority (106/108, 98.1%) of genotyped samples were collected from pediatric patients, whereas only 1.9% (2/108) from adults. The RVA strain detected in the 2 adult patients (a 25-year-old immunocompetent woman and an 88-year-old immunocompromised man) was G3P[8]. In pediatric patients, 88.7% (94/106) of RVA positive samples were identified in children aged 0-5 years, whereas 11.3% (12/106) were detected in patients aged 6-12 years. No positive samples were detected in pediatric patients older than 12 years. As shown in *Figure 1*, G1P[8] (28.7%, 27/94) and G3P[8] (27.6%, 26/94) were the most frequently detected RVA strains in children aged 0-5 years, while G9P[8], G12P[8] and G2P[4] accounted for 17.0% (16/94), 14.9% (14/94) and 6.4% (6/94) respectively; the genotypes G9P[4] (3.2%, 3/94), G6P[9] (1.1%, 1/94) and G12P[4] (1.1%, 1/94) were observed sporadically. In children aged 6-12 years, G3P[8] was the most common strain identified (41.6%, 5/12) with G1P[8] (25.0%, 3/12), while G9P[8] and G2P[4] were less frequently detected, each accounting for 16.7% (2/12).

RVA genotyping

Regarding VP7 genotyping detection, the most frequent G-type was G3 (30.6%, 33/108), followed by G1 (27.8%, 30/108), G9 (19.4%, 21/108), G12 (13.9%, 15/108) and G2 (7.4%, 8/108), while G6 (0.9%, 1/108) was identified at a lower rate. Concerning VP4 genotyping, the main P-type was P8 (87.9%, 95/108), whereas P4 (11.1%, 12/108) and P9 (0.9%, 1/108) were observed in a minority of cases.

Table 2 - *Genotypes of RVA characterized in the period September 2015-April 2018.*

Genotype	*Number of genotyped strains (%)*
G3P[8]	33 (30.6%)
G1P[8]	30 (27.8%)
G9P[8]	18 (16.7%)
G12P[8]	14 (12.9%)
G2P[4]	8 (7.4%)
G9P[4]	3 (2.8%)
G6P[9]	1 (0.9%)
G12P[4]	1 (0.9%)
Total	108 (100.0%)

Eight G and P type associations were observed. G3P[8] and G1P[8] were the most detected strains, with a rate of 30.6% (33/108) and 27.8% (30/108), respectively, followed by common RVA genotypes G9P[8], G12P[8] and G2P[4] that circulate at the lowest rate, whereas uncommon RVA genotypes such as G9P[4], G6P[9] and G12P[4] were observed sporadically (*Table 2*).

RVA monthly distribution

The analysis of RVA monthly distribution revealed an RVA infections peak in the winter-spring period in 2016, 2017 and 2018, followed by a rapid decrease in the frequency of cases during the summer, reaching the lowest amount in October 2016 and 2017 (*Figure 2*). RVA G3P[8] was mainly observed in the winter-spring period in 2016 and 2017. The G1P[8] genotype was frequently detected between September 2015 and January 2016, whereas it was occasionally detected between March 2016 and January 2018. Subsequently, the majority of G1P[8] type rapidly increased between February 2018 and April 2018. Other common strains, such as the G9P[8] genotype, were observed at a low rate between February and May 2016, with an increase in the number of cases in the period February-August 2017, while the G12P[8] genotype circulated

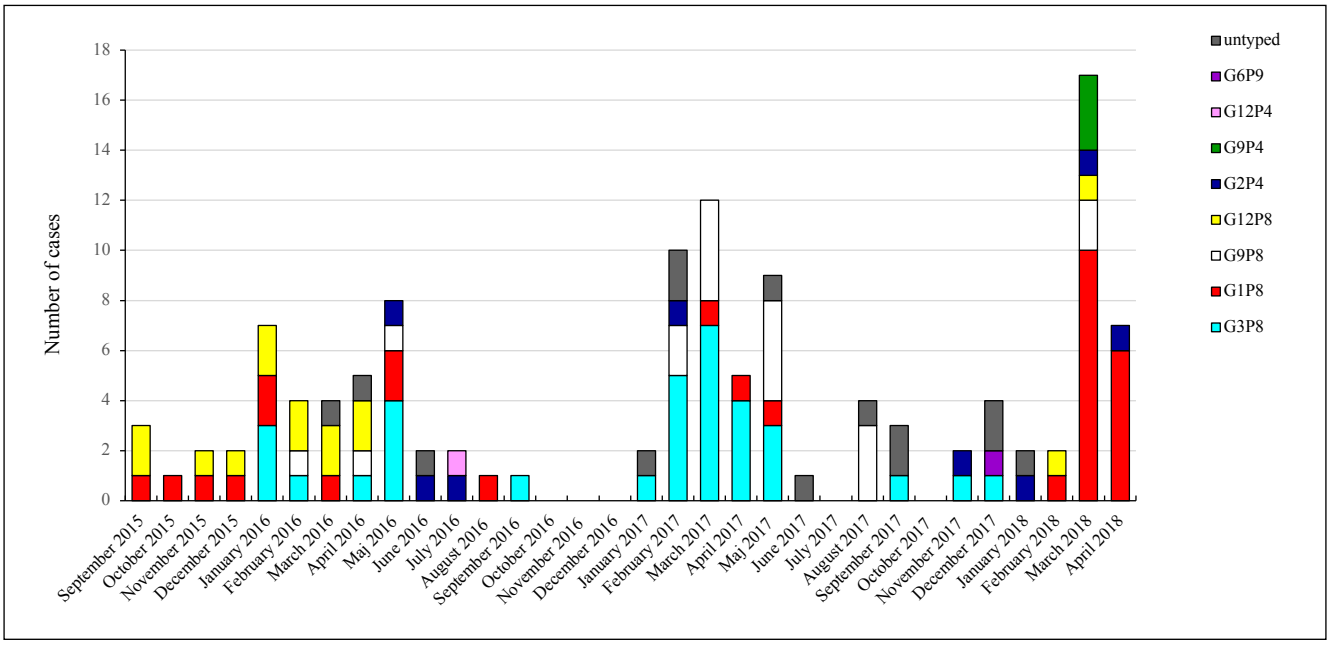

Figure 2 - *Monthly distribution of RVA genotypes in the period September 2015-April 2018.*

constantly from September 2015 to April 2016 and in February and March 2018, and the G2P[4] genotype showed sporadic incidence from May 2016. G9P[4], G6P[9] and G12P[4], uncommon RVA genotypes, were observed sporadically.

DISCUSSION

There are about 15,000 hospitalizations due to rotavirus gastroenteritis in Italy each year (Vitale *et al*., 2013). In the Lombardy Region (Northern Italy, 10 million inhabitants), 32,944 children ≤5 years were hospitalized with acute-gastroenteritis from 2005 to 2011, and 50.8% of cases were related to Rotavirus infection (Pellegrinelli *et al*., 2015). In the present study, we report the seasonal distribution and molecular characterization of RVA in pediatric and adult hospitalized patients in the period September 2015-April 2018 in Pavia province, Lombardy Region. In agreement with previous Italian studies (Biscaro *et al*., 2018; Pellegrinelli *et al*., 2015; Ianiro *et al*., 2019) the majority of RVA cases were pediatric patients with a median age of 29 months, while only a small portion of cases were adult patients. As observed in a previous Italian study (Ruggeri *et al*., 2011), the highest number of RVA cases occurred in the winter-spring period, while few RVA infections were detected during the rest of the year, reaching the lowest amount in autumn. The molecular characterization of RVA strains confirmed that the "common" strains G3P[8], G1P[8], G9P[8] and G2P[4] were the most frequently detected, with a prevalence of 82.4%. While this is in line with the data from the Italian Surveillance Network Rota-Net-Italia (Ruggeri *et al*., 2011) and an Italian study conducted by Ianiro *et al.* (2017), the prevalence of "common" strains G3P[8], G1P[8], G9P[8] and G2P[4] in our study (*Table 2*) was highly dissimilar to what reported in Northern Italy in the period 2006-2009 (Ruggeri *et al*., 2011) as well as to the prevalence in another province of the Lombardy Region in 2015-2016 (Biscaro *et al*., 2018). In particular, the most prevalent strain, G3P[8], which showed a frequency rate of 30.6% in our survey, was detected in 2.8% (Ruggieri *et al*., 2011) and of 7.5% (Biscaro *et al*., 2018) of samples, respectively. Unexpectedly, the strain G4P[8], detected in previous Italian surveillance studies with a prevalence of 10.2% and 16.85% in Italy (Ruggeri *et al*., 2011; Ianiro *et al*., 2019) and around 6.0% in the north of Italy (Ruggeri *et al*., 2011; Biscaro *et al*., 2018), was not detected in our study. In contrast, the "common" strain G12P[8] and the "uncommon" G9P[4], G6P[9], G12P[8] were detected only in pediatric patients aged 0-5 years.

The huge variability of prevalence and the diversity in RVA genotypes circulation among different Italian regions and between years have been already observed (Ruggieri *et al.*, 2011); nevertheless, the reasons for this variability are currently unexplained. It has been hypothesized that the strains predominance and their re-emergence in a particular geographic area and in a certain period could be associated with residual persistence in healthy carriers, animal reservoirs and in the environment and to the immune pressure previously established in the population (Ruggieri *et al.*, 2011).

As outlined in the last report of the 5th European expert meeting on RVA vaccination, RVA vaccination has been shown to be extremely valuable for reducing the number of gastrointestinal infections caused by RVA and related mortality in children worldwide, including induction of the herd effect, protecting unimmunized individuals, when introduced into routine vaccination programs (De Hoog *et al*., 2018). Furthermore, in all European countries where RVA vaccination was offered, a substantial reduction of hospitalizations due to RVA gastroenteritis, as well as emergency room and primary visits, has been observed (Karafillakis *et al*., 2015). RVA vaccination coverage in Italy is still low (8%) and not homogeneous among the different Italian regions (range: 0-40%), which started the programs at different times (Costantino *et al.*, 2018). In Italy, universal RVA vaccination was introduced for the first time in 2013 in the Sicily Region, with vaccination coverage lower than 50% after 4 years and obtaining a significant reduction of hospitalization due to RVA gastroenteritis (Costantino *et al.*, 2018).

As described in the study conducted by Ruggieri et al. (2011), our data confirm that common RVA strains are the main genotypes circulating in the Lombardy Region, providing valuable information before the introduction of universal vaccination in the region.

In conclusion, this study highlights the variety of RVA strains circulating in Northern Italy in hospitalized patients in a period of three years, emphasizing distinct patterns of distribution in different age groups and between years. More surveillance studies are needed to increase the knowledge of the dynamics of RVA strains distribution and emergence.

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Competing interests

The authors have no conflicts of interest to declare.

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