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Research paper

Uncommon G9P[4] group A rotavirus strains causing dehydrating diarrhea in young children in Italy

Giovanni Ianiro[a,*], Claudia Recanatini[b], Marcello M. D'Errico[b], Marina Monini[a], the RotaNet-Italy Study Group[1]

[a] Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità, Rome, Italy
[b] Faculty of Medicine, Department of Biomedical Sciences and Public Health, Polytechnic University of Marche, Ancona, Italy

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ABSTRACT

Group A rotaviruses (RVA) are one of the major cause of acute gastroenteritis (AGE) in young children, being responsible for up to 250.000 deaths worldwide, mostly in developing countries. The two outer capsid proteins VP7 (glycoprotein, G-genotype) and VP4 (protease-sensitive protein, P-genotype) are the basis for the binary RVA nomenclature. Although at least 36 G-types and 51 P-types of rotavirus are presently known, most RVA infections in humans, worldwide as well as in Italy, are related to six major G/P combinations: G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8].

In November 2016, in the framework of the Italian 2016/17 rotavirus surveillance season, a total of 22 rotavirus-positive samples from hospitalized children presenting AGE symptoms were collected in a small area of Central Italy (Ancona, Marche). After genotyping, 3 samples presented the G9P[4] genotype.

In order to better understand the origin of these uncommon RVA strains causing dehydrating diarrhea in three children, the strains RVA/Human-wt/ITA/AN18/2016/G9P[4], RVA/Human-wt/ITA/AN19/2016/G9P[4] and RVA/Human-wt/ITA/AN22/2016/G9P[4] were subjected to nucleotide sequencing of all the 11 gene segments to define their genomic constellation.

Nucleotide sequencing revealed that the genomic constellation of the three strains was G9-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2, highlighting human origin for all the gene segments investigated.

The molecular characterization of RVAs and the continue monitoring of their circulation is needed to better define the epidemiology of these pathogen and to detect the emergence of viral variants presenting a high spreading potential in humans in the post-vaccination era.

1. Introduction

Group A rotaviruses (RVA) are important etiological agents of acute dehydrating gastroenteritis (AGE) in infants and young children worldwide, and are responsible for approximately 250,000 deaths each year, mostly in developing countries (Tate et al., 2016). The RVA genome is composed of 11 segments of double-stranded (ds) RNA, coding for six structural proteins (VPs) and five or six non-structural proteins (NSPs) (Estes and Cohen, 1989). RVAs present the possibility to generate progeny with a high diversity through processes of both genetic drift and genetic shift. Genetic drift is due to the accumulation of point mutations generated by the viral RNA-dependent RNA polymerase; genetic shift represents exchange of one or more segments in the RVA genome between different strains, during the co-infection of the same host. Gene reassortment is one of the most important source of RVA genetic diversity (Desselberger, 1996; Parra et al., 2004; Ramig, 1997). Since the discovery of RVA, many studies have defined the protective role of antibodies against RVA infection and the existence of neutralization epitopes on both outer capsid proteins, VP7 and VP4 (Greenberg et al., 1983; Hoshino et al., 1985; Kalica et al., 1981b; Offit and Blavat, 1986; Taniguchi et al., 1985). RVA has a binary classification system based on nucleotide sequence similarities of VP7 and VP4, which determine the G- and P-genotype respectively. Currently 36 G-genotypes and 51 P-genotypes have been described (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg). To allow closer molecular characterization of RVA evolution interspecies transmissions and reassortment events the traditional classification scheme has been extended to the entire genome specifying genotypes for all the 11 genome segments (Matthijnssens et al., 2008b). On the basis of whole RVA genome sequencing, a specific genotype for each of

* Corresponding author at: Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità, Rome 299 00161, Italy.
E-mail address: giovanni.ianiro@iss.it (G. Ianiro).
[1] The list of members of the RotaNet-Italy Study Group who contributed data is given in the appendix

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11 genes was assigned. Using this classification system two major human non-G and non-P RVA genogroup constellations were detected: genogroup 1 (Wa-like constellation, G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1) and genogroup 2 (DS-1 like, G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2) (Kalica et al., 1981a; Matthijnssens and Van Ranst, 2012). In addition, a minor genomic constellation has been reported rarely: genogroup 3 (AU-1-like constellation, G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H3 (Matthijnssens and Van Ranst, 2012).

With an increasing amount of full RVA genome sequences available worldwide, it has become clear that most of human RVA strains belong to one of these three genomic constellations, showing high sequence similarity in all their genes to one either prototype strains Wa, DS-1, or AU-1 (Mingo et al., 2017). Different studies demonstrates that RVA genomes tend to maintain preferred and stable genome segment constellations (gene sets) (McDonald et al., 2009a; McDonald et al., 2009b; McDonald and Patton, 2011).

A national surveillance network for RVA AGE is active in Italy since 2007, comprising the Istituto Superiore di Sanità, the Ministry of Health, hospitals and universities. Epidemiological surveillance has shown that G-types G1-, G9 and G12 in association with P-types P[4] and P[8] are the most prevalent cause of RVA associated gastroenteritis in Italy (Ruggeri et al., 2011; Delogu et al., 2015; De Grazia et al., 2014; Biscaro et al., 2018), confirming previous studies conducted worldwide (Gentsch et al., 2005). Since 1995, the G9 genotype has emerged in human hosts worldwide, and is considered one of the major common human RVA genotypes, largely circulating in Italy (Ianiro et al., 2013). Despite this increased circulation, the G9 genotype is not included in any present vaccine composition, although anti-RVA vaccination seems to induce effective protection against RVA AGE due to G9 RVA strains (Linhares et al., 2008; Ruiz-Palacios et al., 2006; Vesikari et al., 2006).

During the 2016/17 RVA surveillance season in Italy, a total of 22 rotavirus-positive stool samples were collected from hospitalized children presenting AGE symptoms in a small area of Central Italy (Ancona, Marche), and among these, three uncommon G9P[4] RVA strains were detected. This unusual G/P combination has been previously reported as prevalent in many countries worldwide, becoming the major RVA genotype affecting the pediatric populations of South-East Asia, Japan and Central America (Afrad et al., 2013; Chitambar et al., 2014; Quaye et al., 2013; Reesu et al., 2013; Yamamoto et al., 2015). In addition, a sporadic case has been detected in the US in 2010 (Lewis et al., 2014).

The G9P[4] genotype has never been detected before in Italy, and the previously reported spreading power of this strain in the human pediatric population highlighted the need for a deep molecular characterization of the 11 genomic segments, in order to define the constellation of these strains, and to understand their origin and molecular epidemiology.

2. Materials and methods

2.1. Clinical cases and RVA identification

Cases were three young children aged 0–5 years and presenting acute gastroenteritis symptoms. All patients lived in the same urban area and were admitted to the main public hospital of Ancona (Central Italy) in 2016, with a diagnosis of dehydrating gastroenteritis. The routine microbiological screening revealed the presence of group A rotavirus as causative agents of the gastrointestinal symptomatology. Therefore the cases were reported to the RotaNet-Italy surveillance system and stool samples were sent to the Istituto Superiore di Sanità of Rome for genotyping (Ruggeri et al., 2011). Clinical information were obtained from the provided surveillance questionnaires, filled out by the pediatric units, in compliance with the Informed Consent Agreement.

2.2. Reverse transcription-polymerase chain reaction and nucleotide sequencing

Total RNA was extracted from 140 µl of 10% fecal suspensions in distilled water, using the Viral RNeasy Mini Kit (Qiagen/Westburg, Segrate, Italy), according to the manufacturers' instructions. RNA was eluted in 60 µl of RNase-free water, and stored at −80 °C until use.

After an initial step of denaturation, the viral RNA was subjected to retro-transcription (RT) using the Invitrogen Superscript III reverse transcriptase kit (Life Technologies, Monza, Italy) with a single cycle at 37 °C for 60 min and 95 °C for 5 min. The obtained DNA was then used as template for PCR amplification of VP7 (primers Beg9-End9) and VP4 (primers Con3-Con2) segments (Gentsch et al., 1992; Gouvea et al., 1990). The reactions were performed with the Invitrogen Platinum Taq kit (Life Technologies), following the manufacturers' instructions. RVA genotyping was carried out by a multiple semi nested-PCR using a mixture of primers specific for G- and P-types, as previously described (Gentsch et al., 1992; Iturriza-Gomara et al., 2004).

Retro-transcription and all PCR reactions were accomplished following slightly modified EuroRota-Net protocols (http://www.eurorota.net/docs.php). PCR products were visualized on 2% agarose gel, stained with GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA), for genotype assignment.

For nucleotide sequences analysis, RT-PCR reactions included primers specific for each gene investigated (Esona et al., 2009; Matthijnssens et al., 2008a), using an annealing temperature of 50 °C for all reactions. A 3 min elongation step was used to obtain VP1-4, VP7 and NSP2–5 amplicons, whereas for VP6 and NSP1 elongation was protracted for 6 min.

Nucleotide sequencing of amplified genes was performed at Eurofins Genomics (Ebersberg, Germany), using primers used for PCR. Genotypes for each of the 11 genomic segments were obtained using the RotaC genotyping tool (http://rotac.regatools.be/) (Maes et al., 2009).

2.3. Nucleotide and amino acid sequences analysis

Nucleotide sequences obtained were analyzed and corrected with ChromasPro2.23 software (Technelysium, Queensland, Australia). Nucleotide and amino acid sequence similarity searches were performed using the BLAST (Basic Local Alignment Search Tool) server on the GenBank database of the NCBI (National Center for Biotechnology Information, National Institute of Health, Bethesda, MD).

The deduced amino acid sequences alignments were performed with the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/).

Multiple sequence alignments and phylogenetic tree construction were performed with the MEGA6 software (Tamura et al., 2013), applying the Maximum-Likelihood (ML) method. The substitution model for each tree was obtained by ModelTest from MEGA6. The sequences obtained in this study are available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers: from MG981251 to MG981261 for strain RVA/Human-wt/ITA/AN18/2016/G9P[4]; from MG981262 to MG981272 for strain RVA/Human-wt/ITA/AN19/2016/G9P[4]; from MG981273 to 981,283 for strain RVA/Human-wt/ITA/AN18/2016/G9P[4]. The study strains are referenced below as “AN Italian strains”.

3. Results

3.1. Epidemiological investigation

This study reports the molecular characterization of three uncommon G9P[4] RVA strains (RVA/Human-wt/ITA/AN18/2016/G9P[4], RVA/Human-wt/ITA/AN19/2016/G9P[4] and RVA/Human-wt/ITA/AN22/2016/G9P[4]) circulating in a small area of Central Italy in 2016. RVA strains belonging to this uncommon genotype were detected sporadically only in 2016 in the peninsular Italy, and were not detected