Infection, Genetics and Evolution 29 (2015) 118–126

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Increased detection of G3P[9] and G6P[9] rotavirus strains in hospitalized children with acute diarrhea in Bulgaria

Zornitsa Mladenova [a,b,*,1], Sameena Nawaz [b,1], Balasubramanian Ganesh [c], Miren Iturriza-Gomara [d]

[a] National Center for Infectious and Parasitic Diseases, Sofia, Bulgaria
[b] Public Health of England, London, United Kingdom
[c] National Institute of Cholera and Enteric Diseases, Kolkata, India
[d] Institute of Infection and Global Health, University of Liverpool, Liverpool, United Kingdom

ARTICLE INFO

Article history:
Received 11 June 2014
Received in revised form 23 September 2014
Accepted 12 November 2014
Available online 20 November 2014

Keywords:
Rotavirus
Human strain
Reassortant
Zoonotic transmission
Rotavirus vaccine

ABSTRACT

Rotavirus severe disease in children is now vaccine-preventable and the roll-out of vaccination programs globally is expected to make a significant impact in the reduction of morbidity and mortality in children <5 years of age. Rotavirus is also a pathogen of other mammals and birds, and its segmented RNA genome can lead to the emergence of new or unusual strains in human population via interspecies transmission and reassortment events. Despite the efficacy and impact of rotavirus vaccine in preventing severe diarrhea, the correlates of protection remain largely unknown. Therefore, rotavirus strain surveillance before, during and after the introduction of immunization programs remains a crucial for monitoring rotavirus vaccine efficacy and impact. In this context, molecular characterization of 1323 Bulgarian rotavirus strains collected between June 2010 and May 2013 was performed. A total of 17 strains of interest were analyzed by partial sequence analysis. Twelve strains were characterized as G3P[9] and G6P[9] of potential animal origin. Phylogenetic analysis and comparisons with the same specificity strains detected sporadically between 2006 and 2010 revealed the constant circulation of these unusual human strains in Bulgaria, although in low prevalence, and their increased potential for person-to-person spread.

1. Introduction

Rotaviruses (RVs) are the leading cause of acute viral gastroenteritis in children under 5 years of age. The total number of RV diarrhea cases has considerably declined since 2001, but RVs still account for the death of 420–494 thousand children ⩽5 years old, as half of the cases occur in 5 developing countries (Tate et al., 2012; Parashar et al., 2009). In 2006 two RV vaccines were approved, and since then they have been included in many national immunization calendars around the globe. The implementation of RV vaccination program in many countries has revealed the dramatic decrease of the RV hospitalizations and economic benefits connected with this (Rha et al., 2014; Linhares and Justino, 2014; Nakagomi et al., 2013a; De Oliveira et al., 2013; Raes et al., 2011). However, in countries without RV vaccine included into the national immunization programs, RVs are still responsible for high morbidity and mortality (Yen et al., 2011; Mladenova et al., 2011).

RVs are classified as a genus of the family *Reoviridae*. The genome of RVs, enclosed in a triple-layered protein capsid, consists of 11 double-stranded RNA segments which code for a total of 12 viral proteins, six structural (VP1–VP4, VP6, VP7) and six nonstructural (NSP1–NSP6) (Estes and Kapikian, 2007). RVs are classified into groups A to G based on epitopes of the VP6 intermediate capside protein, as RVs group A (RVA) have the major human and veterinary health impact. The two outer capsid proteins of RVA, VP7 (glycosylated, or G-type) and VP4 (protease-sensitive, or P-type), elicit neutralizing antibody responses, and may have a role in homotypic protection. For this reason G and P-types are the basis for the traditional binary nomenclature of RVA (Hoshino et al., 1985). To date, at least 27 G and 37 P genotypes have been defined in human, mammals and birds according to molecular-genetic diversity of the VP7 and VP4, respectively (Matthijnssens et al., 2011a). At least, 12 G types (G1–G6, G8–G12 and G20) and 15 P types (P[1]–P[11], P[14], P[19], P[25], and P[28]) have been detected in RVA-infected humans. Recently, a novel genotyping classification scheme based on all 11 genome segments has been

* Corresponding author at (formerly): National Reference Laboratory of Enteroviruses, Department of Virology, National Center of Infectious and Parasitic Diseases, 44A, Stoletov Blvd., Sofia 1233, Bulgaria. Tel.: +359 2 931 23 22x247; fax: +359 2 943 30 75.
E-mail address: zornitsavmbg@yahoo.com (Z. Mladenova).

[1] The ZM and SN were equally contributed to the investigation reported in the present study and to the preparation of the draft.

http://dx.doi.org/10.1016/j.meegid.2014.11.011
1567-1348/

proposed providing valuable information on the RV genetic diversity and relationships among human and animal strains (Matthijnssens et al., 2008a). However, the traditional classification of RVAs into G/P genotype combination is still used in broad and long-term surveillance studies, as among human RVAs these two are the proteins displaying the greatest level of diversity and also the genes encoding these two proteins are the one involved in the highest rates of reassortment events when compared to the remaining nine segments (Patton, 2012).

Although many epidemiological studies have revealed that 5 G/P combinations, including G1, G3, G4 and G9 in combination with P[8], or G2P[4], are predominant worldwide, human RVAs with other genotypes (G5, G8, G10, G12, P[6], P[9]) have increasingly been detected in several countries, predominantly in Africa, Asia and South America (Mijatovic-Rustempasic et al., 2014; Ahmed et al., 2013; Nakagomi et al., 2013b; da Silva et al., 2011; Wu et al., 2011; Esona et al., 2010; Iturriza-Gómara et al., 2004a). Furthermore, there is increasing recognition that many of these less common human RV strains may originate through interspecies transmission often accompanied by single or multiple reassortment events (Ianiro et al., 2013; Papp et al., 2013; Steyer et al., 2010, 2013; Mladenova et al., 2012). These RVA stains, in particular those that through reassortment have acquired the ability for efficient person-to-person transmission, have the potential to emerge as epidemic strains. In fact, this is believed to be the mechanism that lead to the global emergence of G9P[8] strains in the mid-nineties (Page et al., 2010a; Bozdayi et al., 2008; Rahman et al., 2005; Steyer et al., 2005; Iturriza-Gómara et al., 2004b; Ramachandran et al., 2000), and to the widespread circulation of G8P[6] strains in various African countries (Nakagomi et al., 2013a; Page et al., 2010b; Todd et al., 2010; Matthijnssens et al., 2006).

Rotavirus strain surveillance started in Bulgaria in 2005 with the aim to study the natural diversity of RVA circulating in the population prior to the introduction of rotavirus vaccines. In excess of 2,000 RV strains were genotyped in Bulgaria between 2005 and 2010 (Mladenova et al., 2010; Mladenova et al., 2012). In addition, 1323 RV strains collected between June 2010 and May 2013 were genotyped, and 17 strains were investigated further as they failed to be G genotyped or were found as having scarce circulation in the country. Here we describe the characterization of these strains though partial genome sequencing and phylogenetic analysis which revealed a potential for sporadic zoonotic transfer of RVAs in Bulgaria.

2. Materials and methods

2.1. Samples

Between June 2010 and May 2013, 4,431 diarrhoeal stool samples from children and adults admitted to hospitals in 5 regions in Bulgaria were tested for the presence of RV antigen using a commercial immunochromatography test (Rota-Strip, Coris BioConcept, Belgium) or enzyme-linked immunosorbent assay (RIDASCREEN Rotavirus, R-Biopharm, Germany) at Virology Laboratory of Specialized Hospital for Active Treatment of Infectious and Parasitic Diseases and at National Reference Laboratory of Diarrheal Viruses, NCIPD, Sofia. None of the patients tested had received RV vaccine. A total of 1323 RV-positive samples were selected for G and P genotyping on the basis of the age of the patient (children between 2 months and 8 years old), the location and month of isolation, and the availability of the samples. Genotyping was performed as previously described (www.eurorota.net/ last accessed April, 2014).

Seventeen RV strains of interest were further characterized by partial genome sequencing. In addition, three G3P[9], two G3P[8], two G6P[9] and two G6P[8] RVA strains detected in Bulgaria between 2006 and 2010, which were characterized by partial genome sequence analysis in previous investigations, were also used for analysis.

2.2. Nucleotide sequencing

Rotavirus VP7 and VP4 amplicons, obtained with the oligonucleotide primers VP7-F, VP7-R, VP4-F, and VP4-R (Iturriza-Gomara et al., 2011) were purified using commercial spin column method (Qiagen GmbH, Hilden, Germany) and sequenced directly using the same primers and an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems, Inc., Foster city, California, USA).

2.3. Sequence and phylogenetic analysis

The chromatograms were checked using BioEdit V.5.0.9. Comparisons between Bulgarian sequences and other RV VP7s and VP4s available in the GenBank database were made by BLAST server (www.ncbi.nlm.nih.gov/Blast). Multiple VP7 and VP4 sequence alignments and phylogenetic analysis were done using Clustal W in the BioEdit software and MEGA v5.10 analytical package (Tamura et al., 2011), respectively. The phylogenetic trees were constructed using the Neighbor-joining method by bootstrapping with 1000 replicates, and phylogenetic distances were measured by the Tajima–Nei model, implemented in the MEGA software. Clusters were defined when bootstrap values were >85%. Nucleotide sequences of BG strains obtained in this study has been deposited in GenBank database under accession numbers KM590348–KM590397.

3. Results

A total of 2010 (45.4%) stool samples were positive in RV-screening test, of them 1323 strains were subjected to genotyping. Single G and P types were obtained for 1230 (93.0%) RV isolates, while 57 (4.3%) were mixed infections and 37 (2.7%) were partially typed (G or P untypeable). G1P[8], G2P[4], G4P[8] and G9P[8] represented 96.0% (1181 isolates), 26.3% ($n = 324$), 31.4% ($n = 386$), 23.5% ($n = 289$) and 14.8% ($n = 182$), respectively, of the total of samples fully genotyped. Seventeen isolates of interest were subjected to sequencing. In RT-PCR 6 strains left as G-untypeable, sequence analysis revealed they were with G6P[9] specificity. The G/P genotype combination of the other 11 were confirmed as G3P[9] (6 isolates) or G3P[8] (5 strains) (Table 1). In addition, Bulgarian strains with G6 ($n = 4$), G3 ($n = 5$), and P[9] ($n = 5$) characterized previously were used for the comparative analysis.

3.1. Analysis of VP7 of the G3 strains

A total of 11 G3 RV strains were sequenced. Nucleotide sequencing of the G3 strains revealed that 5 of them were in combination with typical human P genotype, P[8] (BG1299/2010, BG1344/2010, BG1548/2010, BG1596/2010, and BG347/2012), while the rest 6 strains were having P[9] genotype (BG1253/2010, BG1262/2010, BG1328/2010, BG1431/2010, BG1271/2012, and BG1511/2012) (Fig. 1). Five additional Bulgarian G3 strains were included in phylogenetic analysis for comparison. The Bulgarian G3s segregated into two different lineages, all but two G3P[8] strains, BG1299/2010 and BG1344/2010, clustered within the typical human G3 lineage, represented by the prototype strain RVA/Hu-tc/USA/P/1974/G3P[8]. The Bulgarian strains within this cluster showed homology of 99.5–99.8% at nt and 100% at the deduced amino acid (aa) levels, and were 97% and 98% similarity at the nt and aa levels, respectively to the human prototype strain