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Complete genetic characterization of human G2P[6] and G3P[6] rotavirus strains

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ABSTRACT

During the 2008–2009 rotavirus season, 10 G3P[6] rotavirus strains were isolated for the first time in Belgium, while an outbreak of G2P[6] strains occurred in the USA in 2005–2006. Partial sequencing of the 11 genome segments of the 10 Belgian G3P[6] strains revealed a clonal origin. Two of these strains, and a G2P[6] strain representative of the American outbreak, were selected and sequenced completely to analyze their evolutionary relationships. Genetic analysis revealed that all strains possessed a DS-1-like genotype constellation. The 2 Belgian G3P[6] strains showed >99% sequence identity at the nucleotide level and the American G2P[6] strain was phylogenetically closely related to the Belgian P[6] strains. These data suggest that reassortment(s) involving VP7 occurred recently, and that the prevalence of DS-1-like P[6] rotavirus strains need to be closely monitored because the currently licensed RVA vaccines contain neither the P[6] genotype nor strains with a complete human DS-1 genotype constellation.

1. Introduction

Human group A rotaviruses (RVA) are a major cause of severe gastroenteritis and cause significant morbidity and mortality in infants and young children worldwide (Parashar et al., 2006; Tate et al., 2012). The genus rotavirus belongs to the *Reoviridae* family, and is further subdivided into 8 rotavirus species or groups A to H, based on antibody responses mainly against VP6, and a VP6-based sequence classification using a 53% amino acid cut-off value (Matthijnssens et al., 2012). RVAs possess a genome of 11 double-stranded RNA segments encoding six structural (VP1–VP4, VP6 and VP7) and six non-structural (NSP1–NSP6) proteins. Historically, RVAs have been further classified in different ways based on antigenic differences in VP6 (subgroups), characteristic RNA profiles (electropherotypes) or RNA–RNA hybridization (genotype constellation) (Estes and Kapikian, 2007). Three human genotype constellations, represented by reference RVA strains Wa (genotype constellation 1), DS-1 (genotype constellation 2) and AU-1 (genotype constellation 3) have been established (Matthijnssens et al., 2008a; Nakagomi and Nakagomi, 1989).

Based on sequence similarities of the glycoprotein VP7 and the protease-sensitive VP4, which form the outer capsid of the virus particle and elicit neutralizing antibodies, a widely used binary classification system exists (Estes and Kapikian, 2007; Matthijnssens et al., 2008a). At least 35 different P-types have been identified for VP4, while for VP7 at least 27 different G types have been isolated from humans, mammals and birds (Matthijnssens et al., 2011). The G-genotypes G1, G2, G3 and G4, in combination with P[8] or P[4], are globally most frequently associated with RVA infection (Banyai et al., 2012; Matthijnssens et al., 2009; Santos and Hoshino, 2005). The prevalence of two other genotypes, G9 and G12, strongly increased in the human population since 1995 and 2000, respectively, and human G9 and G12 RVA strain have now spread across the entire globe (Matthijnssens et al., 2010a, 2008b). RVAs bearing genotype P[6] in association with a variety of G-genotypes have been detected at high frequencies in Africa (Adah et al., 2001; Cunliffe et al., 2010; Santos and Hoshino, 2005; Steele and Ivanoff, 2003; Steele et al., 1995; Todd et al., 2010), but the relative frequency of P[6] strains outside Africa is rather low, although outbreaks of P[6] strains have been described in developed countries (Clark et al., 2011; Martella et al., 2008; Timenetsky Mdo et al., 1994). P[6] RVAs have been associated with both symptomatic and asymptomatic cases of childhood gastroenteritis and are also regarded as a major P-type in porcine RVAs (Ciarlet et al., 1995; Hoshino et al., 1985; Lorenzetti et al., 2011; Martella et al., 2006, 2005). The fact that the P[6] genotype, which is believed to be of porcine origin, is more frequently detected in developing countries is believed to be due to the close proximity in which humans and animals often live in these regions (Matthijnssens et al., 2008b).

The RVA gene segments (encoding VP1, VP2, VP3, VP6, NSP1, NSP2, NSP3, NSP4 and NSP5), have been classified into 9 R-genotypes, 9 C-genotypes, 8 M-genotypes, 16 I-genotypes, 16 A-genotypes,

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9 N-genotypes, 12 T-genotypes, 14 E-genotypes, and 11 H-genotypes respectively, based on specific nucleotide sequence cut-off identity values for each gene segment (Matthijnssens et al., 2011). Recently, a standardized RVA strain nomenclature was proposed by the Rotavirus Classification Working Group (Matthijnssens et al., 2011). Although the extended classification system is a leap forward in the characterization of RVA strains, including the detection of reassortment and interspecies transmission events, there is still need for additional complete RVA genomes to be analyzed, especially those of currently circulating human strains to better understand the dynamics of RVA diversity, evolution, and selective immunologic pressure (Ghosh and Kobayashi, 2011).

In 2006, two RVA vaccines were licensed in many countries around the world. In many of these countries, including Belgium and the USA, RVA vaccination is included as part of the routine vaccination schedule for all infants. One vaccine is a monovalent live attenuated human G1P[8] vaccine (Rotarix™, GlaxoSmithKline Biologicals, Rixensart, Belgium), the other is a pentavalent human-bovine reassortant vaccine (Rotateq™, Merck & Co., Inc., Whitehouse Station, NJ, USA). The latter contains five human genotypes (G1–G4, P[8]) and the bovine genotypes G6 and P[5] into the backbone of the bovine WC3 strain (Ciarlet and Schodel, 2009; Heaton and Ciarlet, 2007; Matthijnssens et al., 2010b). Several other live RVA vaccine candidates are under development including the bovine (UK strain)-human reassortant vaccine (targeting human VP7 genotypes G1-G4, G8 and G9), the natural bovine-human reassortant neonatal 116E (G9P[11]), and the human neonatal RV3 strain (G3P[6]) (Ciarlet and Schodel, 2009).

Because none of the currently licensed RVA vaccines contain the P[6] genotype, it is important to monitor the prevalence of the P[6] genotype in the human population and to understand the overall genetic constellation relationship of P[6] RVA strains to those of the more prevalent RVA strains worldwide that are associated with genotypes P[8] or P[4]. Therefore the aim of this study was to investigate the genetic constellation of contemporary human P[6] RVA strains circulating in developed countries. This study describes the first G3P[6] RVA strains detected in Belgium since the start of our surveillance at the Gasthuisberg University Hospital in 1999 and our Belgian surveillance network in 2007. The complete genome constellation of two representative G3P[6] strains isolated in Belgium, as well as that of a representative G2P[6] strain isolated in the USA during an unusual outbreak of G2P[6] strains in the 2005–2006 RVA epidemic season, were sequenced and compared. Their relationship to other known RVA strains was also investigated.

2. Materials and methods

2.1. Study specimens

During the 2008–2009 RVA season, 10 G3P[6] RVA strains were isolated in children as part of the Belgian RVA surveillance network that has been in place since 2007 (unpublished data). Five of the G3P[6] RVA strains were detected in cases hospitalized in the same hospital and treated by the same physician in April 2009, suggesting a RVA outbreak of G3P[6] RVA strains in the vicinity of Brussels. The remaining cases of G3P[6] RVA infections were detected in the northern and eastern parts of Belgium. After a partial nucleotide sequence analysis of the ten G3P[6] strains, two of the G3P[6] RVA strains, strain RVA/Human-wt/BEL/BE1322/2009/G3P[6] isolated from a two month old infant during the outbreak in Brussels and strain RVA/Human-wt/BEL/BE1498/2009/G3P[6] isolated from a three month old infant in Antwerp, were selected to determine the complete genome sequence.

Early during the 2005–2006 epidemic RVA season in Philadelphia, USA, a limited number of G2P[4] RVA strains were identified but these strains were quickly replaced by an unusual high number (32%) of G2P[6] RVA strains by the end of the RVA season (Clark et al., 2011). All these G2P[6] RVA strains exhibited an identical electropherotype; therefore, one strain RVA/Human-wt/USA/06-242/2006/G2P[6] (kindly provided by Clark, Children's Hospital of Philadelphia, Pennsylvania, USA), isolated from a hospitalized child with gastroenteritis in the Children's Hospital of Philadelphia, Pennsylvania, USA, was selected to determine the complete genome constellation.

2.2. RNA isolation and RT-PCR

Viral RNA was extracted using the QIAamp® Viral RNA Mini kit (Qiagen/Westburg, Leusden, the Netherlands) according to the manufacturer's instructions. Extracted RNA was denatured at 95 °C for 2 min and reverse transcription – polymerase chain reaction (RT-PCR) was performed using the Qiagen® OneStep RT-PCR Kit (Qiagen/Westburg). Used primer sequences are available upon request. The RT-PCR reactions were carried out with an initial reverse transcription step at 45 °C for 30 min, followed by PCR activation at 95 °C for 15 min, 35 cycles of amplification and a final extension of 10 min at 70 °C by using a Biometra T3000 thermocycler (Biometra, Westburg, Benelux). Cycle conditions for the amplification of the smaller genomic segments encoding VP6, VP7, NSP2, NSP3, NSP4 and NSP5 were as follows: 45 s at 94 °C, 45 s at 48 °C and 3 min at 72 °C. For the larger segments encoding VP1, VP2, VP3, VP4, and NSP1 the cycle conditions were 45 s at 94 °C, 45 s at 50 °C and 6 min at 70 °C.

2.3. Nucleotide sequencing

Each of the gene specific PCR amplicons were purified with the MSB Spin PCRapace Kit (Invitek, Berlin, Germany) and sequenced using the dideoxynucleotide chain termination method with the ABI PRISM™ BigDye Terminator Cycle Sequencing Reaction kit (Applied Biosystems, Foster City, CA, USA) with an ABI PRISM™ 3130 automated sequencer (Applied Biosystems). The sequencing was performed with the same forward and reverse primers as for the RT-PCR. Primer walking sequencing was performed to cover the complete genome on both strands. To obtain the complete nucleotide sequence of strains BE1322, BE1498 and 06-242 the 5′ and 3′ terminal sequences of the 11 genomic segments were determined using a modified RACE technique as previously described (Matthijnssens et al., 2006a).

2.4. Nucleotide and protein sequence analysis

The chromatogram sequencing files were analyzed using Chromas 2.3 (Technelysium, Queensland, Australia), and contigs were generated using SeqMan (Lasergene version 7.0; DNASTAR, Madison, WI, USA). Nucleotide and protein sequence similarity searches were performed using the National Center for Biotechnology Information (NCBI, National Institutes of Health, Bethesda, MD, USA) BLAST (Basic Local Alignment Search Tool) server on GenBank database release 143.0 and nucleotide identities were calculated using the P-distance (Altschul et al., 1990). Multiple sequence alignments were calculated using ClustalX 2.0.12 (Larkin et al., 2007). The sequence alignment was manually edited in Mega 4.0.1. (Tamura et al., 2007).

2.5. Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 4.0.1. software, based on the nucleotide sequences of the different RVA gene segments available in GenBank (Tamura et al., 2007). Genetic distances at the nucleotide level were calculated using the Kimura-2 parameter. The