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A B S T R A C T


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 (Adal 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,
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 (Rotateg™, 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 (Clariet and Schodl, 2009; Heaton and Clariet, 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 (G3P[11]), and the human neonatal RV3 strain (G3P[8]) (Clariet and Schodl, 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 Gastrohuisberg 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


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 PCRpacle Kit (Invitrek, 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
2.6. Accession numbers

GenBank accession numbers (VP1-VP4, VP6, VP7, NSP1-NSP5) for each individual genomic segment are: JF640812-JF640822 (RVA/Human-wt/USA/06-242/2006/G2P[6]), JF640823-JF640833 (RVA/Human-wt/BEL/BE1322/2009/G3P[6]) and JF640834-JF640844 (RVA/Human-wt/BEL/BE1498/2009/G3P[6]).

3. Results


To investigate the genetic relatedness among the ten Belgian G3P[6] strains, 106 gene segments (for a single strain the VP1, VP2, VP3 and NSP5 gene segment could not be amplified) were partially determined (at least 650 nt per gene segment, data not shown). Because these nucleotide sequences showed very high similarities across the 11 genome segments (>99.4%), two RVA strains isolated in different Belgian cities (BE1322 and BE1498) were selected for complete genome analysis.

3.2. Genotype constellation of RVA strains BE1322, BE1498 and 06-242

The complete genomes of RVA strains BE1322, BE1498 and 06-242 were sequenced and the deduced amino acid sequences were determined. Both complete genomes of the Belgian G3P[6] RVA strains, BE1322 and BE1498, contained 18,613 nucleotides and 5798 deduced amino acids. The entire genome of the American G2P[6] RVA strains 06-242 contained a total of 18,612 nucleotides, encoding a total of 5798 amino acids. The difference of one nucleotide between the American and Belgian strain is due to a deletion of one thymine in a homopolymer located in the 5’ non-coding region of NSP1.


3.3. Phylogenetic relationship of RVA strains BE1322, BE1498 and 06-242 to other RVA strains

Phylogenetic trees of all eleven genome segments were constructed (Figs. 1–3). Overall, the VP1–VP3, VP6, NSP1–NSP5 of both Belgian G3P[6] strains showed a very high degree of sequence identity (>97% at the nucleotide level) with the RVA G2P[6] strain isolated in the USA. Our data show that despite being isolated in two different continents and approximately 3 years apart, the Belgian G3P[6] RVA strain and the American G2P[6] strain are very similar (with the VP7 gene segment being the main differentiator), indicating that reassortment between strains with different G-genotypes have occurred recently. For the VP1, VP2, VP3, VP6, NSP1, NSP2, NSP3, NSP4 and NSP5 encoding genome segments of RVA strains 06-242, BE1322 and BE1498, a close phylogenetic clustering was found with human strains possessing DS-1-like genotypes, respectively in the R2, C2, M2, I2, A2, N2, T2, E2 and H2 genotypes (Figs. 1–3). Although all nine of the above mentioned genome segments belong to the DS-1-like genotype 2, the genetic identity between RVA strains BE1322, BE1498, 06-242 and other recently isolated DS-1-like RVA strains varied. For example, there is a very close relationship between BE1322, BE1498, 06-242 and recently isolated strains from Belgium (B1711), the USA (LB2744, LB2772 and LB2764), Germany (GER1H–09), China (TB-Chen), Bangladesh (RV176-00, RV161-00, N26-02, MMCC, MMC88), South-Africa (GR10924/99 and 3203WC), Malawi (1473), Kenya (D205) and the Democratic Republic of Congo (DRc88, DRc86), for the genome segments encoding VP2 (93.5–98.9% identical at the nucleotide level), NSP1 (96.0–98.4%), NSP3 (96.2–98.3%) and NSP5 (95.7–99.8%). For the genome segments encoding VP1, VP3, VP6, NSP2 and NSP4 of RVA strains BE1322, BE1498, 06-242, there is a mixed picture, with high nucleotide identities to some of the above-mentioned strains (VP1, 98.3% between BE1322, BE1498, 06-242 and GR10924/99; VP3, 98.2% between 06-242 and GR10924/99; VP6, 99.5% between 06-242 and B1711; NSP2, 97.4% between 06-242 and MMCC6; NSP4, 97.8% between BE1498, 06-242 and MMC6), and low nucleotide identities to other strains (VP1, 84.1% nucleotide identity between 06-242 and 1473; VP3 80.9% between BE1322, BE1498, 06-242 and D205; VP6 84.8% between 06-242 and TB-Chen; NSP2, 85.3% between 06-242 and TB-Chen; NSP4, 86.5% between BE1498 and GER1H–09).

The VP4 encoding genome segments of the investigated strains were found in a cluster containing only human P[6] RVA strains, distinct from the porcine P[6] RVA reference strain RVA/Pig/tc/USA/Gottfried/1983/G4P[6]. A dendrogram of the P[6] genotype showing the relationship of RVA strains BE1322, BE1498 and 06-242 with other complete VP4 nucleotide sequences is illustrated in Fig. 1. The 3 RVA strains are most closely related (97.6–97.9% nucleotide identity) to the human B1711 strain (G6P[6]), isolated from a 13-month-old child admitted to the university hospital in Leuven, Belgium, with severe gastroenteritis after returning from a trip to Mali (Mathijsens et al., 2008c; Rahman et al., 2003). The three strains also showed high identity (ranging from 95.7% to 97.0% at the nucleotide level) with other recent P[6] RV strains isolated in the Democratic Republic of Congo (DRc86, G8P[6]), Malawi (MW23, G8P[6]), South Africa (GR10924, G9P[6]), and Bangladesh (RV161, RV176, N26). The nucleotide identity of the two P[6] Belgian strains (BE1322 and BE1498) and the American strain (06-242) to the P[6] VP4 gene of the candidate RVA vaccine RV3 was 95%.
The VP7 encoding genome segment of the Belgian strains possessed the G3 specificity, a genotype which is normally associated with the P[8] genotype constellation (McDonald et al., 2009). The VP7 sequence of RVA strains BE1322 and BE1498 showed a 96.1–96.2% nucleotide sequence identity with the old reference strain RVA/Human-tc/USA/P/1974/G3P1A[8]. Recently, partial VP7 and VP4 sequences of G3P[6] RVA strains isolated from Ethiopia became available in Genbank (accession numbers JF909035-JF909042). These strains showed a 98.2–98.8% nucleotide sequence identity with the VP4 and VP7 sequences of the Belgian G3P[6] strains. Comparing the VP7 gene segment of the American RVA strain 06-242 to other human G2 RVA strains, revealed that they shared 98.3%–98.5% nucleotide sequence identity with the following Asian G2 RVA strains: RVA/Human-XX/IND/SC27/XXXX/G2P[4], RVA/Human-tc/TWN/1993/G2P[X] and RVA/Human-tc/JPN/KO-2/2000/G2P[4] (Iijima et al., 2006).

### 4. Discussion

Human P[6] RVA strains are not frequently detected in developed countries such as Belgium and the USA were the most common genotypes are G1, G3, G4 and G9 in combination with P[8] and G2 in combination with P[4]. However, the prevalence of the different G/P genotype combinations often fluctuate from place to place and season to season (Iturriza-Gomara et al., 2011, 2009; Matthijnssens et al., 2009; Santos and Hoshino, 2005). For example the prevalence of the G3 genotype detected during RVA surveillance at the Gasthuisberg University Hospital, Leuven, Belgium (1999–2007) and in Belgium (from 2007 onwards) was always low but varied over the years, with a major increase in the 2002–2003 RVA season (24.4%) and a persistent increase during the 2003–2004 RVA season (31.9%). None of these Belgian G3 RVA strains were associated with the P[6] genotype. After the 2004–2005 season, in which the G3P[8] was prevalent in 17.2% of the cases, the G3 genotype disappeared for three consecutive RVA seasons, but reappeared during the 2008–2009 season (Rahman et al., 2005; Zeller et al., 2010). In the 2008–2009 RVA season, 13% (n = 10) of the detected G3 strains were found in combination with the P[6] genotype. In 2009–2010, only 1.8% of the RVA positive samples were genotyped as G3, whereas the G3 genotype predominated again during the 2010–2011 RVA season (31.7%), although none of these G3 RVA strains were found in combination with the P[6] genotype (unpublished results).

For 7 out of 10 cases infected with G3P[6] RVA strains the age was known, and all were below 3 months of age (17 days to 3 months). This could suggest that these G3P[6] RVA strains have a higher tendency to cause gastroenteritis in neonates, as was previously described for P[6] strains in Bangladesh (Kilgore et al., 1996).

As previously reported, 3 different G3 phylogenetic lineages were found to co-circulate in Belgium in 2008–2009, two of which were associated with P[8], and one more distantly related G3 lineage with P[6] (Zeller et al., 2011). This suggests that the Belgian G3P[6] RVA strains were most likely not derived from a reassortment of a local G3P[8] RVA strains with a strain carrying the P[6] genotype, but rather that this G3P[6] lineage was imported. The first G3P[6] RVA case in Belgium was detected in February 2009 in Antwerp. During the following months, G3P[6] strains were sporadically detected throughout the country with a small outbreak around Brussels in April 2009. As expected, all Belgian G3P[6] RVA strains (G3 lineage 3) were highly similar (>99% nucleotide identity) based on the partial nucleotide sequences of all eleven genome segments, indicating a clonal origin.

Full-genome sequencing showed that G3P[8] RVA strains preferably have internal gene constellations belonging to genotype 1 (McDonald et al., 2009). However, this study describes the first...
the detected G2 strains combined with the P[6] genotype (Clark et al., 2011; Hull et al., 2011; Payne et al., 2009). The American RVA strain 06-242 is the first strain isolated from this outbreak that was completely characterized in this study.

Since the start of the RVA genotype surveillance at the Gasthuisberg University Hospital in 1999 and our Belgian surveillance network in 2007 only one P[6] strain, B1711, has been completely characterized (Rahman et al., 2005; Matthijnssens, 2008). RVA
strain B1711 is a G6P[6] strain isolated from a 13 month old child that was hospitalized in Belgium after a trip to Mali (Matthijnssens et al., 2008c). These data indicate that the strain was imported from Mali to Belgium. B1711 showed high similarities to the VP4, VP2, VP6 and all non-structural proteins of the Belgian BE1322 and BE1498 RVA strains, as well as the American 06-242 RVA strain. Most likely, the Belgian G3P[6] strains were also imported from abroad. The fact that the P[6] sequences of the strains described in this study are most similar to strains isolated in Africa (more specific from Ethiopia), underscores a potential African origin. However, to answer this kind of question in the future, it is important that more sequence information on RVA strains from developing countries becomes available.

Noteworthy is the fact that human P[6] strains combine with a wide variety of G-genotypes (G2, G3, G4, G6, G8, G9, G12) and can have either a Wa-like or a DS-1 like background (Esona et al., 2010).

Fig. 3. Phylogenetic dendrograms based on the full-length nucleotide sequences of RVA NSP1, NSP2, NSP3, NSP4, and NSP5 genes. The bootstrap values (1000 replicates) are shown at the branch nodes (values < 70% not shown). The strains from this study are marked with a circle.
The strains analyzed in this article are of special interest in the light of the current known epidemiology of P[6] strains worldwide. Until now, P[6] RVA strains are frequently detected in the African continent and in India and Bangladesh, were an incidence up to 43% of P[6] RVA strains has been reported in the past (Cunliffe et al., 2010; Ramachandran et al., 1996; Santos and Hoshino, 2005; Todd et al., 2010; WHO, 2011). Strains with P[6] specificity have also sporadically been detected at very low frequencies among cases of RVA gastroenteritis in Ireland (Cashman et al., 2011), France (Bon et al., 2000), Italy (Arista et al., 2003), the UK (Cubbit et al., 2000; Iriturria-Gomara et al., 2000), USA (Clark et al., 2011; Griffin et al., 2000) and the Netherlands (Widdowson et al., 2000). Data from EuroRotaNet, the European rotavirus surveillance network, comprising 18 European countries, also reported the presence of P[6] RVA strains in numerous European countries (Iriturria-Gomara et al., 2011, 2009). It is, however, unclear if the increased prevalence of P[6] genotypes in more industrialized regions is related to RVA vaccine introduction, improved detection methods, or if this is attributable to normal genotype fluctuations. Continued surveillance and more genetic information of currently circulating P[6] strains will be essential to answer this question in the future. The prevalence of the P[6] genotype, in particular within the context of the DS-1-like genotype constellation, needs to be closely monitored because the success of vaccination programs can be influenced by genetic heterogeneity of human RVA strains. Both currently licensed RVA vaccines, Rotarix™ and RotaqTeq™, do not contain strains with the P[6] genotype or strains with a complete DS-1-like human genotype constellation (Matthijssens and Van Ranst, 2012). It would be interesting to see how effective the licensed RVA vaccines, and the human neonatal candidate vaccine RV3 (G3P[9]), which possesses a Wa-like genotype constellation (Ripponger et al., 2010), are in protecting against disease brought on by G3P[6] or G2P[6] with a complete DS-1-like genotype constellation.

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