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During the 2009 national group A rotavirus (RVA) surveillance, five unusual strains of the human G8P[6] genotype were detected in Brazilian Indian children with acute gastroenteritis. The aim of this study was to carry out sequence analysis of the two outer capsid proteins (VP4 and VP7) and the inner capsid protein (VP6) of the G8P[6] strains detected in order to provide further information on the genetic relationship between human and animal RVA. A total of 68 stool samples, collected in Mato Grosso do Sul during 2009, were tested for RVA using ELISA, following by reverse transcriptase-PCR and sequencing. RVA infection was detected in 7.3% of samples (5/68). The IAL-RN376 G8 sequence shares a clade with bovine and human strains, displaying highest nucleotide identity to African human strains DRC86 and DRC88, followed by African bovine strain NGR89b8. IAL-RN376 and IAL-RN377 P[6] sequences showed highest identity to human strain R330 from Ireland, and a close genetic relationship to African fruit bat RVA strain KE4852/07. Strains IAL-RN376 and IAL-RN377 display genogroup I VP6 specificity and the I2 genotype, and share high nucleotide identities with human strains B1711, 272-BF and 06-242, and moderate identities with bovine (RUBV81, 86 and K9-1) and porcine (HP140) strains. This study suggested that a reassortment between bovine and bat RVA strains could have occurred in animal host(s) preceding the transmission to humans. In the indigenous population, zoonotic transmission is probably fairly frequent as the inhabitants live in close contact with animals under conditions of poor hygiene.

INTRODUCTION

Group A rotavirus (RVA) is an important aetiological agent of gastroenteritis in humans and animals. RVA usually exhibits host species restriction; however, a number of atypical RVA strains isolated from humans and animals share genetic and antigenic features of virus strains from heterologous species (Khamrin et al., 2006; Matthijnssens et al., 2008a), suggesting that interspecies transmission or reassortment between animal and human viruses can occur (Timenetsky et al., 1997; Kapikian et al., 2001; Estes & Kapikian, 2007; Matthijnssens et al., 2011; Luchs et al., 2012). The two outer capsid proteins, VP4 and VP7, allow classification into P and G genotypes, respectively, and sequence analysis of the genes that code for these proteins is useful for gathering epidemiological information and tracing the origin of unusual RVA strains (Cook et al., 2004; De Grazia et al., 2007; Steyer et al., 2008; Martella et al., 2010; Luchs et al., 2012; Mukherjee et al., 2013; Mullick et al., 2013). Strains with uncommon VP7 and VP4 genes, regarded as animal-like, have been identified sporadically in humans and have acquired epidemiological relevance in some geographical areas (Iturriza-Gómar et al., 2004). The VP6 protein is the major structural component of the virion; it is highly antigenic and immunogenic, and might play a role in inducing protective immunity (Estes & Kapikian, 2007).

During 2009 national RVA surveillance, five unusual strains of the human G8P[6] genotype were detected in Brazilian Indian children with acute gastroenteritis. RVA strains with G8 specificity are usually detected in calves (Martella et al., 2010). However, human G8 strains have been reported globally, including in Brazil (Santos et al., 1998; Volotão et al., 2006; Bányai et al., 2009; Esona et al., 2009; Page et al., 2010; Ghosh et al., 2011; Middley et al., 2012; Mukherjee et al., 2013), and have often been characterized as originating from zoonotic transmissions. The P[6] genotype can circulate reasonably efficiently in the human population (Martella et al., 2006) and has been detected in Brazil since the 1980s (Timenetsky et al., 1994; Carmona et al., 2004, 2006). Nevertheless, recent findings...
strongly suggest interspecies transmission of RVA P[6] strains between pigs and humans (Martella et al., 2006).

The aim of this study was to carry out sequence analysis of the two outer capsid proteins (VP4 and VP7) and the inner protein (VP6) of the G8P[6] strains detected in order to obtain further information on the genetic relationship between human and animal RVA.

**RESULTS**

RVA infection was detected in 7.3% of samples (5/68). The median age of the children was 5.6 months and 80% were males. Three RVA samples exhibited a short profile by PAGE, and two were negative. Using reverse transcriptase (RT)-PCR genotyping of the VP7 and VP4 genes with panels of primers specific for various human G and P types, and genotyping of the VP6 gene, all samples were characterized as G8-P[6]-I2 (Table 1). One sample of G8 specificity (IAL-RN376), and two samples of P[6] and I2 specificities (IAL-RN376 and IAL-RN377) were selected for sequencing.

Fig. 1 shows a comparison of amino acid sequences of the six antigenic regions A–F (Estes & Kapikian, 2007) between strain IAL-RN376 and reference RVA strains belonging to the G8 genotype. The antigenic regions A–F of strain IAL-RN376 clearly support its classification as genotype G8. There was 100% amino acid homology in all antigenic regions between strain IAL-RN376 and the human strain DRC86 isolated in the Democratic Republic of Congo in 2003 (Fig. 1). The alignment of amino acid sequences deduced from the VP7 gene revealed amino acid substitutions in strain IAL-RN376 inside the variable region C (aa 120–130) at position 122T→V, region D (aa 143–152) at position 146A→I, region E (aa 207–220) at position 218V→I, and region F (aa 233–242) at position 237I→V. Amino acid substitutions were also observed outside VP7 hypervariable regions in strain IAL-R2638: 26I→V, 65T→M, 116V→I, 139F→V and 186S→A (Fig. 1). VP7 hypervariable regions B (aa 87–101), C (aa 120–130), E (aa 207–220) and F (aa 233–242) were the most conserved regions in all strains analysed, whilst regions A (aa 39–50) and D (aa 143–152) were the most variable. The VP7 protein of strain IAL-R2803 had two potential N-linked glycosylation sites located at aa 69 and 238 (Fig. 1).

A comparison of the nucleotide and deduced amino acid sequences of strain IAL-RN376 with those of other published human and animal G8 RVAs revealed that the VP7 gene of strain IAL-RN376 had the highest identity to human strains DRC86 (98.9% nt; 99.6% aa) and DRC88 (98.7% nt; 99.3% aa), both isolated in the Democratic Republic of Congo in 2003, followed by human strain RV1122 (97.7% nt; 97.3% aa) isolated in Spain in 2009. Strain IAL-RN376 also exhibited high nucleotide and amino acid identity to the simian strain KY1646 (98% nt; 98.6% aa) and the bovine strain NGRBg8 (95.7% nt; 97% aa), both also isolated on the Africa continent. The high identity shared between strain IAL-RN376 and animal strains was stressed in the phylogenetic tree (Fig. 2).

The comparative sequence analysis revealed that the bovine G8 strain NGRBg8 displayed higher nucleotide identity with human strains (96.1–99.8% nt; 94.4–99.6% aa) and simian strain KY1646 (97.2% nt; 97.7% aa) than with other bovine strains (82.2–86.5% nt; 94.4–96.3% aa). In fact, bovine strain NGRBg8 showed highest genetic identity with human strain HMG035 (99.8% nt; 99.6% aa), isolated in Nigeria in 1999–2000. Bovine strain NGRBg8 also displayed high similarity with other Brazilian human strains: R291 (96.1% nt; 96.7% aa), 5353 (96.9% nt; 97.7% aa), 5877 (96.9% nt; 97.7% aa) and 5664 (96.8% nt; 97.7% aa). When compared with additional human G8 strains used in the present study, strain IAL-RN376 had 84.2–97.8% nt and 93.4–98.3% aa homology, including the human Brazilian strain R291 (95.8% nt; 96.3% aa). The lowest nucleotide and amino acid identity was observed between strain IAL-RN376 and bovine strain Cody 1801 (82.6% nt; 93.4% aa) (Fig. 2).

G8 lineages were suggested previously by Fukai et al. (2004) and Pietsch et al. (2009). On the basis of the VP7 phylogenetic tree, six different lineages were identified, named Lineages I–VI (Fig. 2). Lineage I comprised a single bovine strain (Cody 1801) isolated in the USA. Lineage II was occupied by animal strains and an environmental strain (U259.96) detected in Switzerland. Lineage III consisted of three human strains and a single bovine strain (678) from Scotland. Lineage IV consisted of two human strains (6746 and SI-885). Lineage V comprised four human strains and one bovine strain (A5) isolated in Thailand. Lineage VI was the most complex. This lineage consisted of 14 human strains, including strain IAL-RN376 detected in this study.

<table>
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Fig. 1. Deduced amino acid sequence of the VP7 protein of human strain IAL-RN376 and a selection of G8 rotaviruses. The VP7 antigenic regions A–F are indicated. The N-linked glycosylation sites at positions 69 and 238 are indicated by asterisks. Isolates of each strain are indicated.
Fig. 2. Nucleotide-based phylogenetic relatedness of the IAL-RN376 RVA G8 VP7 gene (indicated by the arrow) to other selected human and animal G8 strains. The RVA strains of particular interest are highlighted in bold. The neighbour-joining tree of the partial VP7 nucleotide sequence was generated with MEGA 4. Reference G8 strains were obtained from GenBank. Accession number, species, isolate, country and year are indicated for each strain. Roman numerals I–VI represent six different lineages. The scale indicates the number of divergent nucleotide residues. Percentages of bootstrap values are shown at the branch nodes.
and other Brazilian human strains (R291, 5664, 5353 and 5877). In addition, Lineage VI comprised the simian strain KY1646 and bovine strain NGRBg8, both detected in Africa (Fig. 2).

Fig. 3 shows the deduced amino acid sequence of the VP4 (subunit VP8*) of human strains IAL-RN376 and IAL-RN377, and representative VP4 amino acid sequences of the RVA P[6] genotype. The potential cleavage sites, arginine (R) 230, 240 and 246, were maintained in strains IAL-RN376 and IAL-RN377. The arginine at position 230 in strains XI99-468 and BJ-CR4916, isolated in China, were substituted by a lysine (K). The highly conserved cysteine (C) at residue 215, and prolines (P) at residues 68, 71, 224 and 225, were maintained in strains IAL-RN376 and IAL-RN377 (Fig. 3).

Comparative analysis of the deduced amino acid sequences of the IAL-RN376 and IAL-RN377 VP4 fragment (VP8* subunit) showed that the variable region between aa 71 and 204 was fairly conserved among all strains analysed, confirming the classification of strains IAL-RN376 and IAL-RN377 as genotype P[6]. Within the VP8* subunit variable region, substitutions had occurred in strains IAL-RN376 and IAL-RN377 at positions 135N and 146N (Fig. 3). The amino acid substitution at position 135N was also shared by human strains R330 (Ireland), KY6950 (Kenya) and BP1231/02 (Hungary), and by porcine strains detected in Brazil (BRA898/07-Po) and Italy (221/04-19, 221/04-20 and 221/04-21). Amino acid substitutions were also observed outside the VP4 hyper-variable region in strains IAL-RN376 and IAL-RN377: 30S and 255S (Fig. 3).

The overall nucleotide sequence identity between the VP4 gene (VP8* fragment) of strains IAL-RN376 and IAL-RN377 and those of cognate P[6] sequences ranged from 80 to 99.6%. The VP4 sequence of strains IAL-RN376 and IAL-RN377 showed the highest identity to the human R330 strain, isolated in Ireland in 2003–2006, at 99.6% (99.2% aa) and 99.4% (98.4% aa) on the nucleotide level, respectively. Comparison of the IAL P[6] RVA sequences showed 99.8% nt (99.2% aa) similarity among them.

In addition, genetic analysis of the VP4 gene revealed that two Brazilian P[6] human strains (HST435 and HST369) and one human South Korean strain (KMR720) clustered with an African straw-coloured fruit bat (Eidolon helvum) RVA strain (KE4852/07) detected in Kenya in 2007 (97.6% nt; 96.1–96.5% aa). However, the deduced amino acid sequence of VP4 of the bat KE4852/07 strain showed the highest identity to human strain 6782 (96.4% nt; 94.6% aa) at the nucleotide level, respectively. When the strains IAL-RN376 and IAL-RN377 and Brazilian human strains HST435 and HST369 were compared with each other, similarities of 96.4–96.5% nt (96.9–97.6% aa) were revealed. The VP4 sequences of human strains IAL-RN376 and IAL-RN377 were also compared with those of strains representing porcine P[6] types (BRA898/07 and Gottfried), and the nucleotide identities ranged from 80 to 87.9% (80–89.2% aa) (Fig. 4).

On the basis of the VP4 phylogenetic tree, the five different lineages described previously (Martella et al., 2006) were identified, i.e. Lineages I–V (Fig. 4). Strains IAL-RN376 and IAL-RN377 clustered in Lineage I. Lineage I consisted exclusively of human strains isolated from several countries, except for the bat strain KE4852/07 isolated in Kenya in 2007. Lineage II comprised a single porcine strain (Gottfried) isolated in the USA in 1975. Lineage III consisted of two porcine strains (JP3-6 and JP29-6) from Japan. Lineage IV was occupied by human strains isolated in Hungary (BP1198/98, BP1338/99 and BP271/00). Lineage V consisted exclusively of porcine strains isolated in Italy in 2004. Curiously, the porcine strain BRA898/07 detected in Brazil in 2007 did not cluster in any lineage (Fig. 4).

Comparative analysis of the deduced amino acid sequences of the fragment of VP6 known to correlate subgroup (SG) specificity (Itrrriza-Gomara et al., 2002) allowed characterization of strains IAL-RN376 and IAL-RN377 as genogroup I (SGI) (data not shown). Phylogenetic analysis of the VP6 genes revealed that strains IAL-RN376 and IAL-RN377 clustered within I2, which is typical of strains belonging to the DS-1 genome constellation (Nakagomi et al., 1989). The VP6 gene of strains IAL-RN376 and IAL-RN377 share high nucleotide identities of 93.5–98.5% (80.9–96% aa) with those of human I2 strains, and 99.1% (97% aa) between them. Although strains IAL-RN376 and IAL-RN377 were assigned to the I2 genotype, the VP6 gene of strains IAL-RN376 and IAL-RN377 exhibited extremely low nucleotide and amino acid sequence identities (83.2–83.8% nt; 54.3–54.9% aa) to the prototype strain DS-1, and clustered separately by phylogenetic analysis (Fig. 5).

Strains IAL-RN376 and IAL-RN377 also shared moderately high nucleotide identities with three bovine RVA I2 strains: RUBV81 (90.9–91.2% nt; 72.1–72.5% aa) and 86 (90.3–90.6% nt; 71.4–71.8% aa) isolated in India, and KJ9-1 (91.7–92% nt; 75–75.4% aa) isolated in South Korea. In addition, moderate nucleotide identities were observed between strains IAL-RN376 and IAL-RN377 and the porcine strain HP140 I2 detected in India (90–90.3% nt; 70.1–70.5% aa). The VP6 genes of strains IAL-RN376 and IAL-RN377 clustered with strain B171 isolated in Belgium in 2002 (97.6–98.2% nt; 93.2–96% aa), strain 272-BF isolated in Burkina Faso in 2010 (97.3–98.2% nt; 92.2–95% aa) and strain 06-242 isolated in the USA in 2006 (97.6–98.5% nt; 93.2–96% aa), all previously described human RVA P[6] strains (Fig. 5).
The bat RVA strain KE4852/07 belongs to a novel VP6 genotype designated I15 (Esona et al., 2010), and low sequence identities were found when compared with P[6] sequences of both animal and human origin (68.5–79.4 % nt; 33.3–49 % aa). Comparison of the partial VP6 nucleotide and amino acid sequences of strains IAL-RN376 and IAL-RN377 with bat strain KE4852/07 also showed low levels of identity (76.8–77.1 % nt; 42.5–43.3 % aa) (Fig. 5).
Fig. 4. Nucleotide-based phylogenetic relatedness of IAL-RN376 and IAL-RN377 RVA P[6] VP4 genes (indicated by the arrows) to other selected human and animal P[6] strains. The bat strain KE4852/07 (■) is indicated. The RVA strains of particular interest are highlighted in bold. The neighbour-joining tree of the partial VP4 nucleotide sequence was generated with MEGA 4. Reference P[6] strains were obtained from GenBank. Accession number, species, isolate, country and year are indicated for each strain. Roman numerals I–V represent five different lineages. The scale indicates the number of divergent nucleotide residues. Percentages of bootstrap values are shown at the branch nodes.
DISCUSSION

The present study detected human RVA strains with unusual G8P[6] combinations in Brazilian Indian children with acute gastroenteritis. Human genotype G8 strains have been reported globally, including in Europe (Cooney et al., 2001), South America (Santos et al., 1998; Montenegro et al., 2007; Gómez et al., 2010), Australia (Bishop et al., 2001) and Asia (Kang et al., 2002). However, in Africa, they are of epidemiological importance and have been reported at frequencies as high as the globally common G3 or G4 strains (Gentsch et al., 2005; Santos & Hoshino, 2005; Page et al., 2010).

This study demonstrated that the G8 Brazilian strain IAL-RN376 shared a high level of RNA homology with...
Democratic Republic of Congo strains (Matthijnssens et al., 2006). The close relationship between Brazilian and African G8 strains had already been observed previously (Montenegro et al., 2007; Gómez et al., 2010; Page et al., 2010). The Brazilian and African populations have always been interconnected due to a significant immigration flow (Gómez et al., 2010), including the forced migration (slave trade) during the nineteenth century, leading to the exchange of RVA strains (Gómez et al., 2010). Nevertheless, the question of how these strains were transported from one continent to another, e.g. by international air travel, imported or exported food, animal products (Page et al., 2010) and infected children during the incubation period, remains unresolved.

The G8 genotype also has a wide distribution in animal species, and has been reported in pigs, horses and cattle (Cooney et al., 2001; Cao et al., 2009). Therefore, the origin of G8 strains in the human population has been investigated in recent years and interspecies transmission from a bovine source has been suggested (Adah et al., 2003; Matthijnssens et al., 2006; Cao et al., 2009; Esona et al., 2009; Ghosh et al., 2011). Comparative sequence analyses showed that the Brazilian G8 strain IAL-RN376 displays a close genetic relationship to the bovine African G8 strain NGRBg8. In fact, human African G8 RVA strains DRC88 and DRC86 also showed nucleotide sequence identity with the same bovine G8 strain NGRBg8 (Matthijnssens et al., 2006). The available data on circulating bovine RVA strains in Brazil have indicated the G8 genotype in herds in the Midwest region (Alfieri et al., 2004). Indigenous populations and animals often share the same source of water, increasing the chance of animal–human transmission. Therefore, it was not too surprising to detect genotype G8 strains, known to circulate in bovine herds, in Brazilian Indian children living in this region. Unfortunately, none of the animals from the sampling site were screened for the presence of RVA.

In addition, strain IAL-RN376 G8 displayed a close genetic relationship with the simian G8 strain KY1646. However, strain KY1646 did not have an animal origin. This strain was collected from a vervet monkey (V1888) infected experimentally with an unpassaged RVA strain obtained from the stool of a 6-year-old child (JMM-9/11) hospitalized with acute gastroenteritis at MP Shan Hospital in Nairobi, Kenya in 1999 (Chege et al., 2005). G8 strains with animal genetic characteristics are rare and do not persist for long periods in human populations (Page et al., 2010).

The potential VP7 N-linked glycosylation site was located at aa 69 in strain IAL-RN376, which tends to be conserved among RVA strains (Martella et al., 2003). In addition, human strain IAL-RN376 had a second potential glycosylation site at aa 238, like most bovine and human G8 strains (Gouvea et al., 1990; Okada & Matsumoto, 2002). Glycosylation of residue 238 could have far-ranging effects on the immunogenicity, and has previously been shown to reduce neutralization of animal G11 RVA strains by hyperimmune sera and MAbs (Ciarlet et al., 1997).

The amino acid substitution at position 146[^1] T→Y occurred inside the major antigenic site, region D (aa 143–152). The amino acid substitution at position 218[^2] T→T occurred inside region E (aa 207–220), which is spatially very close to region D (Estes & Kapikian, 2007). The precise impact of amino acid changes cannot be predicted from sequence information alone (Zeller et al., 2012) and studies attempting to correlate intragenotypic nucleotide differences with antigenic differences are extremely important (Jin et al., 1996; Hoshino et al., 2004).

Human RVA G8 strains have been found to be associated with a variety of VP4 genotypes, including P[1], P[2], P[4], P[5], P[6], P[8], P[10], P[11] and P[14] (Qian & Green, 1991; Gerna et al., 1994; Cunliffe et al., 1999; Fukai et al., 1999; Fischer et al., 2000; Jagannath et al., 2000; Adah et al., 2001; Kang et al., 2002; Okada & Matsumoto, 2002; Matthijnssens et al., 2006; Page et al., 2010). The ability of RVA strains to reassort freely was also noted for genotype G9 strains (Laird et al., 2003). G8 strains have also shown the ability to reassort repeatedly with RVA strains more ecologically suited to the human gut, i.e. with P[4], P[6] and P[8] specificity (Page et al., 2010). Nonetheless, the fact that genotypes such as G8P[4], G8P[6] and G8P[8] do not remain in circulation suggests that these binary associations may not achieve the fitness required to become a successful human pathogen in some countries, except Africa (Esona et al., 2009; Gómez et al., 2010).

The P[6] genotype has been associated with unusual or novel RVA strains, emerging in naïve populations (Aminu et al., 2010), and it has been speculated that reassortment with the P[6] genotype may provide a mechanism to establish a previously rare G type into a naïve population (Page et al., 2010). Owing to the availability of new sequence data in GenBank, the VP4 gene analysis allowed us to identify a close genetic relationship between African bat KE4852/07 (Esona et al., 2010) and human IAL-RN376 and IAL-RN377 P[6] strains detected in this study. The bat strain KE4852/07 (Esona et al., 2010) was also closely related to strains HST435 and HST369 isolated from neonates in the city of Belem, in the Northern region of Brazil (Mascarenhas et al., 2007). The finding that the bat strain KE4852/07 was nearly identical to human P[6] was described previously by Esona et al. (2010). Bats are being recognized increasingly as reservoir hosts for viruses that can cross species barriers to infect humans and other domestic animals and wild mammals (Calisher et al., 2006; Halpin et al., 2011; Cui et al., 2012; Fagrouch et al., 2012; Marsh et al., 2012; Quan et al., 2013; Smith & Wang, 2013). Fruit bats often live near human habitats (Calisher et al., 2006), and there are various opportunities for bats and humans to come into contact with each other and their respective RVA (Esona et al., 2010). In Indian villages, contact between humans and bats can be even more frequent (Smith & Wang, 2013).

Trypsin cleavage of the VP4 spike protein, which yields two polypeptides, VP8* and VP5*, is required for the activation...
of infectivity (Estes et al., 1981; Gorziglia et al., 1988). The potential VP4 arginine cleavage sites (230, 240 and 246) were maintained in strains IAL-RN376 and IAL-RN377, ensuring infectivity. The four proline residues (68, 71, 224 and 225) are conserved in strains IAL-RN376 and IAL-RN377. These conserved prolines may have a major influence on the conformation of VP4, because proline is known to distort the three-dimensional structure (Gorziglia et al., 1988).

In the study presented here, the analysis of VP6 gene sequences revealed the presence of genogroup I. In general, SGI RVA strains were associated with the P[6] or P[4] genotypes, and SGII RVA strains were associated with the P[8] genotype, demonstrating a genotype-dependent co-segregation pattern (Iturriza-Gómez et al., 2002; Nordgren et al., 2012a, b). We further observed that the VP6 genes of the unusual G8P[6] RVA strains were related closely to VP6 genes of two rare G6P[6] strains: strain B1711 isolated in Belgium in 2002 in a child arriving from Mali (Rahman et al., 2003; Matthijnssens et al., 2008c) and the strain 272-BF isolated in Burkina Faso in 2010 (Nordgren et al., 2012a, b). In addition, the 06-242 strain G2P[6] RVA genotype isolated in the USA in 2006 (Clark et al., 2011; Heylen et al., 2013) was also phylogenetically closely related to strains IAL-RN376 and IAL-RN377, as well as the B1711 and 272-BF G6P[6] RVA strains. This observation could indicate a genetic linkage between VP6 genes from the African and American continents, reinforcing the close relationship observed between Brazilian and African G8 strains. 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 (Heylen et al., 2013).

A significant genetic relatedness of human and animal RVA was also observed in the VP6 gene. Strains IAL-RN376 and IAL-RN377 appeared to share a more common origin with bovine (RUBV81, 86 and K9P-1; nucleotide sequence identities of 90.3–92 %) and porcine (HP140; nucleotide sequence identities of 90–90.3 %) strains than the prototype human strain DS-1 (nucleotide sequence identities of 83.2–83.8 %). Artiodactyl-like human VP6 strains linked to the RVA P[6] genotype have been reported previously in asymptomatic neonates (Ghosh et al., 2013). In addition, it has been speculated that several gene segments of bovine RVA strains, including VP6, have a common ancestor with human DS-1-like RVA strains (Matthijnssens & Van Ranst, 2012).

Lineages among G8 and P[6] strains had already been recognized previously (Fukai et al., 2004; Martella et al., 2006; Pietsch et al., 2009) and, based on the phylogenetic tree, the animal host origin of G8 and P[6] genotypes can be suggested as likely if animal strains are located at the base of the dendrogram. Based on the dendrogram and the amino acid substitutions within variable regions, subclassification of G8 RVA into six different lineages was proposed in the present study. The present data concur with the classification suggested previously by Fukai et al. (2004) (lineages G8a, G8b, G8c and G8d). In contrast, the subclassification proposed by Pietsch et al. (2009) did not comprise any of the G8 lineages observed. In this study, three human strains clustered within the G8 Lineage III (G8c), together with bovine strain 678; four human strains clustered within the G8 Lineage V (G8d), together with bovine strain A5; and 14 human strains clustered within the G8 Lineage VI (not described), together with bovine strain NGRBg8. This is suggestive strongly of a common evolutionary origin of the G8 human and bovine RVAs, and may represent a recent interspecies transmission event between cattle and humans that occurred independently from the event leading to the onset of the G8 Lineage I (G8b). More extensive sequence analysis of G8 strains is needed to establish the lineages correctly.


Based on this study, it is speculated that a reassortment event between bovine G8 and bat P[6] RVA may have occurred in the animal host(s) preceding the transmission to the human host (Esona et al., 2010). However, recent findings suggest strongly that the P[6] strain had a porcine origin (Martella et al., 2006; Matthijnssens et al., 2006) and that humans may have served as a reservoir of transmission of P[6] RVA strains to bats, which can result in anthrozoopozoonotic transmission of RVA genes (Esona et al., 2010). Taken together, these observations suggest that strains IAL-RN376 and IAL-RN377 might be derived from anthrozoopozoonotic transmission; however, with the currently available data it is not possible to confirm the exact direction of these transmission events or to determine unequivocally the origin of these unusual genotypes found in humans (Steyer et al., 2010). It is important to note that the uncommon G8P[6] strains analysed in this study were detected in Brazilian Indian children; in the indigenous population, interspecies transmission is probably fairly frequent as many of the inhabitants live under conditions of poor hygiene, in close proximity with animals, and often share a common source of drinking water. Moreover, children could be more exposed to infection than adults because of their close interactions with pets together with limited hygiene habits characteristic of their age.

The representative isolates, IAL-RN376 and IAL-RN377, had a high level of identity with strains I2 (B1711, 272-BF, 06-242) and G8 (DRC86, DRC88) belonging to the DS-1 genogroup (Matthijnssens et al., 2006, 2008c; Nordgren
et al., 2012a, b; Heylen et al., 2013), suggesting that G8P[6] strains from Brazil belong to viruses of the DS-1 gene constellation. However, genetic characterization of additional genes is necessary to determine the full genetic profile of these strains. Indeed, the determination of complete sequences from all genes, as recommended by the Rotavirus Classification Working Group (Matthijnssens et al., 2008a, b), would have permitted more thorough analyses of sequence data (Esona et al., 2011). The VP6 protein is classified currently into 16 I genotypes (He et al., 2013). It is important to note that the VP6 protein of the bat RVA strain KE4852/07 strain was characterized as a novel VP6 genotype, designated I15 (Esona et al., 2010). Strain KE4852/07 is also the only currently known RVA strain with the I15 genotype. More recently, a G3P[3] strain (MSLH14 strain) was detected from a lesser horseshoe bat (Rhinolophus hippocideros) in China (He et al., 2013). Strain MSLH14 was classified into the I8 genotype and is currently the third known RVA strain with this VP6 genotype (He et al., 2013).

An important limitation of the RVA genomics study between animal and potential zoonotic human cases is presenting: epidemiological background information on potential contact with animals is not generally available nor are animal RVA strains from the area where these patients lived during their illness (Bányai et al., 2010). The present study is no exception and predicting the zoonotic event of a particular RVA strain relies currently only on phylogenetic evidence (Bányai et al., 2010).

In 2006, an attenuated G1P[8] vaccine (Rotarix) was included in the Brazilian Immunization Programme, preventing severe RVA gastroenteritis and inducing a significant reduction in the frequency of RVA detection in children with gastroenteritis (Gurgel et al., 2008). As both VP7 and VP4 play roles in protective immunity (Kapikian et al., 2001; Estes & Kapikian, 2007), knowledge of the distribution of G and P types, including the detection of emerging serotypes, is critical for establishing RVA vaccine programmes (Montenegro et al., 2007). It worth mentioning that other RVA proteins, VP2, VP6, NSP2 and NSP4 epitopes (antibody against which are not neutralizing), are immunogenic and antibodies directed against them are found in most serum samples from convalescing individuals. However, the clinical significance of non-neutralizing RVA-specific antibodies for protection has not been explored fully (Desselberger & Huppertz, 2011). In addition, although a large genetic diversity exists among RVA with respect to the G/P genotypes, the diversity is much smaller on the level of complete genomes, and only Wa and DS-1-like RVA strains are of significant epidemiological importance in humans (Glass et al., 2013; Parashar et al., 2013).

G8 strains had been detected in Brazil before the introduction of the RVA vaccine (Santos et al., 1998; Volotão et al., 2006; Montenegro et al., 2007), and its is important to continue with surveillance studies in the country to have a better understanding of whether such strains represent emerging genotypes, as in some African countries, and whether this should be taken into account in future vaccine developments. In a recent study conducted by Steele et al. (2012), the Rotarix vaccine demonstrated efficacy against severe gastroenteritis caused by diverse circulating RVA types sharing neither G nor P type with the vaccine strain (dually heterotypic RVA types), including G8 specificity (64.4%). In addition, the prevalence of the P[6] genotype, in particular within the context of the DS-1-like genotype constellation, needs to be monitored closely because the success of vaccination programmes can be influenced by the genetic heterogeneity of human RVA strains. Neither of the currently licensed RVA vaccines (Rotarix and RotaTeq) contain strains with the P[6] genotype or strains with the complete DS-1-like genotype constellation (Matthijnssens & Van Ranst, 2012; Heylen et al., 2013).

In conclusion, the findings of this study reinforce the theory that there is a important interaction between RVA of human and animals. Zoonotic studies are hampered by a lack of genome sequencing data of RVA circulating in animals (Jere et al., 2012) and this is especially the case for Brazil. Simultaneous surveillance of animal (including wildlife) and human RVA infections (Martella et al., 2006; Steyer et al., 2008), and accumulation of more sequence data of animal strains (Jere et al., 2012) are vital for the understanding of the zoonosis of these viruses.

**METHODS**

**Faecal samples.** Stool samples from patients with acute gastroenteritis were sent to the Enteric Diseases Laboratory of the Adolfo Lutz Institute, regional reference centre for RVA surveillance, Brazil Ministry of Health, and a member of the Acute Diarrhoea Disease Monitoring Programme (ADDMP), State of São Paulo Department of Health. The aim of the ADDMP is the early detection of diarrhoea outbreaks with a national scope. The samples studied were part of the ADDMP and were detected during 2009 national RVA surveillance (January–December) in Brazilian native children ≤ 3 years with acute gastroenteritis (Table 1).

This study was carried out with convenient surveillance specimens, without inclusion or exclusion criteria, and with no characterization of the participating Brazilian Indian communities. The molecular characterization of RVA genotypes post-Rotarix vaccination was performed without clinical evaluation and the information on RVA vaccination was obtained from medical records. Therefore, the study does not allow assessment of security, immunogenicity or protection provided by vaccination.

The Indian communities were located in the city of Dourados, a municipality of the state of Mato Grosso do Sul, Midwest region, Brazil. Dourados lies near the border with Paraguay (~120 km) and 235 km distant from the state capital (Campo Grande). The main economic activity of the Midwest region is cattle ranching, accounting for ~36% of national production, and the region has the largest herd in Brazil. Beef cattle are the most important, although there are also dairy cattle in the states of the Midwest region (http://www.brascascolac.com/brasil/pecuaria-na-regiao-centroeste.htm).

The state of Mato Grosso do Sul has a large indigenous population that belongs to eight different ethnic groups: Guarani, Kawai, Parataru, Kayapó, Embé, K electromagnetic, Xavante, and Pataxo (IVSBS, 2000).
Terena, Kadiwêu, Kiniakinu, Guatô, Ofaïe and Atikum (Ferreira et al., 2011). The city of Dourados was inhabited previously by Terena, Kaiowa and Guarani ethnicities. However, they were displaced in the 1950s due to agriculture and livestock expansion (http://www.ibge.gov.br/home/). Nowadays, there are two indigenous reservations in the city of Dourados. The Dourados Indian Reservation (‘Terra Indígena Dourados’) was created in 1984, with 3475 ha, located 5 km from the city centre, and with 11000 native inhabitants. The native population are unable to make their living from the land due to lack of sufficient area and appropriate means. The Reservation does not offer hunting and fishing for the inhabitants. In addition, the Reservation is surrounded by large soya plantations and the few natural sources of water are contaminated by urban sewage or pesticides. In 2004, the Panambizinho Reservation was created, located 23 km from the city centre, with 1180 ha, and a current population of 650 native inhabitants (http://www.cpao.embrapa.br/portal/artigos/artigos/artigo17.html, http://www.ibge.gov.br/home/).

Rotavirus detection. A total of 68 stool samples (collected from January to December 2009) were tested for RVA using a commercial immunoenzymatic assay (RIDASCREEN Rotavirus; R-Biopharm), performed according to the manufacturer’s instructions.

PAGE. The RVA migration profiles were analysed by PAGE followed by silver staining of gels (Herring et al., 1982).

Genotyping VP7, VP4 and VP6. RVA dsRNA was extracted by the QIAamp Viral RNA Mini Kit (QIagen) following the manufacturer’s instructions. RT-PCRs for the VP7, VP4 and VP6 genes were performed according to the protocols described previously (Gouvea et al., 1990, 1994; Gentsch et al., 1992; Iturriza-Góñora et al., 2002).

Nucleotide sequencing and analysis. PCR amplicons were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with primers Beg9/End9 for the VP7 gene (1062 bp), Con2/Con3 for the VP4 gene (876 bp) and VP6-F/VP6-R for the VP6 gene (379 bp). Dye-labelled products were sequenced using an ABI 3130 sequencer (Applied Biosystems). Sequencing chromatograms were edited manually using Sequencher 4.7 software. The web-based automated RVA genotyping tool Rotac2.0 (http://rotac.regatools.be) was used to assign the genotype of the study strains (Maes et al., 2009). Sequences generated by manual editing and a set of cognate sequences of human and animal RVA available in GenBank were aligned using the BioEdit sequence alignment editor (version 7.0.5.2) program. Genetic and protein sequence analyses were performed using BioEdit and MEGA 4 (Tamura et al., 2007). Distance matrices were constructed using BioEdit at the nucleotide and amino acid levels to evaluate percentage identity between strains. The Kimura two-parameter substitution model and neighbour-joining method was chosen within MEGA 4 to infer phylogenetic relationships among relevant strains. Nucleotide sequences determined in this study have been deposited in GenBank under the accession numbers JQ693565 for the VP7 gene, JQ693566/JQ693567 for the VP4 gene and KF718287/KF718288 for the VP6 gene.

Ethical approval. Previous Ethics Committee approval was granted by Adolfo Lutz Institute, São Paulo, Brazil (14/05 and 53/05). This was an anonymous unlinkable study and informed consent was not required according to resolution 196/96 concerning research involving humans (Conselho Nacional de Saúde/Ministerio da Saúde, Brasilia, 1996).

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