Distribution of rotavirus VP7 and VP4 genotypes circulating in Tunisia from 2009 to 2014: Emergence of the genotype G12

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INTRODUCTION

Group A rotavirus (RVA) represents the most important aetiological agent of diarrhoeal disease in children ≤5 years old worldwide, with a high mortality impact in Africa and Asia (Estes & Kapikian, 2007; Kotloff et al., 2013; Tate et al., 2012). According to the estimates for 2008, RVA was associated with 453 000 deaths worldwide among children ≤5 years old, mainly (>80%) in countries in Asia and Sub-Saharan Africa (Tate et al., 2012). RVA mortality has been estimated to be 196 000 cases/year in 2011 (Walker et al., 2013).

Abbreviation: RVA, group A rotavirus.

Vaccines represent the best hope for preventing the severe consequences of RVA infection, especially in impoverished regions where resources and access to care may be limited (Madhi et al., 2010). Therefore, the WHO has recommended RVA vaccines for routine immunization of all children worldwide (WHO, 2009). Two RVA vaccines that have been pre-qualified by the WHO, Rotarix (GlaxoSmithKline Biologicals, Belgium) and RotaTeq (Merck & Co., Inc., USA), have been adopted in national immunization programmes in a number of mostly high and middle-income countries, where their effect on rotavirus-related hospital admissions and deaths has been demonstrated (Patel et al., 2012). These vaccines are being introduced in several low-income countries in Africa and Asia, where their
efficacy is lower (Armah et al., 2010; Madhi et al., 2010; Zaman et al., 2010). In Tunisia, Rotarix was introduced on the private market in 2009 according to the recommendation of the WHO but has not yet been included in the national childhood vaccination programme due to its high cost per dose (72 TND/dose; 32.45 EUR/dose). Consequently, the RVA vaccination rate is lower than 5% (Ben Hadj Fredj et al., 2013a).

RVAs belong to the family Reoviridae, subfamily Sedoreovirinae, genus Rotavirus (Carstens, 2010). These viruses are found in both humans and animals, and genetic rearrangement between animal and human rotaviral genomes is a common mechanism for generating RVA diversity (Gentsch et al., 2005; Gouvea & Brandtly, 1995). They are icosahedral, non-enveloped viruses that have a triple-layered capsid with a genome of 11 segments of dsRNA encoding six virion structural proteins (VP1–VP4, VP6 and VP7) and six non-structural proteins (NSP1–NSP6) (Estes & Kapikian, 2007). VP6 is used to classify rotaviruses into groups A–H (Matthijnssens et al., 2012). VP7 is the major neutralization antigen. It is encoded by the ninth genomic segment and is used to classify RVAs into G (glycoprotein) genotypes. VP4 is a minor neutralization antigen encoded by the fourth genomic segment, and it determines the P (protease sensitive) genotypes (Estes & Kapikian, 2007). Recently, a new classification system for RVAs based on the sequences of all the 11 segments of the RVA genome was proposed by the Rotavirus Classification Working Group (RCWG) (Matthijnssens et al., 2008). Currently, based on the nucleotide sequences of outer capsid protein genes, 27 G- and 35 P-genotypes have been reported from humans and animals (Matthijnssens et al., 2011). Among these, some specific G- and P-genotypes are dominant in individual host species (Martella et al., 2010). In human RVAs, 6 G-genotypes (G1–4, G9 and G12) and 3 P genotypes (P[4], P[6] and P[8]) are commonly associated with human infections (Santos & Hoshino, 2005). In addition, several G-genotypes (G5, G6, G8, G10, G11 and G20) and P-genotypes (P[1]–[3], P[5], P[7], P[9]–[11], P[14], P[19] and P[25]), regarded as animal-like strains, have been detected sporadically in humans in different parts of the world (Ben Hadj Fredj et al., 2013b; Gautam et al., 2015; Gentsch et al., 2005; Santos & Hoshino, 2005; Solberg et al., 2009; Tacharoenmuang et al., 2015). At least 73 G/P-genotype combinations of RVAs infecting humans have been described (Matthijnssens et al., 2009). The most common are G1P[8], G3P[8], G4P[8], G9P[8], G2P[4] and G12P[8] (Bánayai et al., 2012; Gómez et al., 2014; Matthijnssens et al., 2010; Rahman et al., 2007; Santos & Hoshino, 2005; Stupka et al., 2012).

Surveillance for RVAs is ongoing in Tunisia through a national RV surveillance group since 1995, providing a unique epidemiological observatory to monitor the evolution of human RVAs (Chouiikha et al., 2007, 2011a). Previous Tunisian studies investigating the VP7 and VP4 genes from human RVA strains showed a wide G/P-genotype diversity. In the present study, we report the molecular characterization of human RVA strains circulating in Tunisia from 2009 to 2014.

**METHODS**

**Materials**

Faecal specimens were collected from children <5 years of age between January 2009 and December 2014. Children were either patients hospitalized in a paediatric unit or outpatients consulting for gastroenteritis. Fourteen distinct hospitals were included in this multi-centre study realized through 11 Tunisian cities: Sousse, Monastir, Mahdia, Sfax, Gabes, Nabeul, Tunis, Kairouan, Jendouba, Beja and Bizerte. The geographical location of the 14 hospitals included in the study covers the coastal region of Tunisia where most of the population is concentrated (Fig. 1).

All samples were immediately screened for RVA detection. RVA-positive samples were stored at −20°C until molecular analysis by PAGE and VP7/VP4 genotyping.

**Methods**

**Detection of RVA antigens.** All samples were processed for rapid diagnosis by ELISA (IDEIA Rotavirus, DAKO Ltd., Glostrup, Denmark) according to the manufacturer’s instructions.

**RNA extraction.** Viral RNA was extracted and purified from 10% faecal suspensions in phosphate-buffered saline using the TRIzol method ( Gibco BRL, Invitrogen, Burlington, Canada).

**PAGE.** All RVA-positive faecal specimens were analyzed by PAGE to characterize the RVA electropherotypes in circulation, as described elsewhere (Steele & Alexander, 1987). Briefly, RNA was extracted from the 10% faecal suspensions previously prepared using phenol-chloroform deproteinization and ethanol precipitation. The extracted RNA was applied to a 3% stacking/10% resolving gel and electrophoresed overnight at 100 V at room temperature, using a discontinuous buffer system. The dsRNA bands were visualized by silver staining, according to the method described by Herring et al. (1982).

**RT-PCR genotyping for VP7 and VP4 genes.** The extracted RNA was used for RT-PCR using specific VP7 and VP4 consensus primer pairs (Das et al., 1994; Simmonds et al., 2008). Viral RNA was heated for 5 min at 97°C to denature the dsRNA segments and was quickly placed back on ice. The denatured RNA was reverse transcribed with AMV reverse transcriptase at 42°C for 45 min, in the presence of primers to the terminal sequences of the VP7 gene (mixture of the primer pairs 9con1/VP7R) (Das et al., 1994)). The cDNA was then amplified with the same primers during 30 cycles, each consisting of heating up to 95°C for 1 min to denature the cDNA, followed by cooling to 42°C for 1 min to anneal the primers and, finally, heating to 72°C for 1 min to extend the strands. The final extension was lengthened for 7 min to promote full-length amplicons.

For the VP7 genotype, two distinct semi-nested PCR reactions were performed. The first reaction was a multiplex PCR using type-specific primers of the VP7 gene, which could detect G1, G2, G3, G4 and G9 (Das et al., 1994). This first PCR was used for all specimens. Samples that remained untypeable after this first VP7-typing assay were further tested with a second PCR assay aiming to detect G12 VP7 specificity. Indeed, to detect the G12 genotype, we optimized a second semi-nested reaction using a G12 specific primer (Samajdar et al., 2006) and, as a positive control, a Tunisian G12 RVA strain which was previously confirmed by PAGE.
sequencing (Ben Hadj Fredj et al., 2013a). The size of the resultant amplicon allows determination of the VP7 genotype.

The VP4 genotype was determined in the same way using an RT-PCR system described by Gentsch et al. (1992). Specific primers (consensus primers VP4F/VP4R) (Simmonds et al., 2008) were used to amplify the VP8* gene which was then differentiated into VP4 genotypes by a cocktail of specific primers designed for the human VP4 genotypes P[4], P[6], P[8], P[9] and P[10] (Gentsch et al., 1992; Iturriza-Gomara et al., 2000; Simmonds et al., 2008).

All amplified products were examined by gel electrophoresis in 2% agarose gels stained with ethidium bromide and viewed under UV illumination.

RESULTS

RVA detection

The total number of samples screened for RVA in the present study was 1127, with 960 (85.2%) collected from inpatients and 167 (14.8%) from outpatients. Globally, a total of 270 (24%) stool specimens were positive for RVA. Among the samples collected from hospitalized children, 24.9% (239) were positive for RVA, whereas 18.5% (31) were positive among the outpatients specimens.
Assessment of the seasonal distribution of RVA diarrhoea showed that the disease most commonly occurred during the coolest season in Tunisia, with peaks observed annually between November and March (Fig. 2).

**PAGE results**

PAGE was performed on all positive samples to examine the genomic diversity of the RVA dsRNA. Among the 270 RVA-positive samples, 182 (67.4 %) were positive by PAGE, as these presented an electropherotype with a characteristic 4-2-3-2 migration pattern typical of RVA. RVA strains generally possess either the short (S) or the long (L) RNA electropherotype. In the present study, nine different electropherotypes could be visualized, six with a long profile (L1–L6) and two with a short one (S1–S2). RVA strains presented long profiles in 173 cases (95 %) and short profiles in 7 cases (3.9 %). Mixed profiles (M) suggesting the presence of dual infections with more than one strain of RVA in a unique sample were detected in two cases (1.1 %) (Fig. 3).

**G-typing results**

Major heterogeneity in the distribution of VP7 genotypes was detected. In total, 267 (98.9 %) of the 270 RVA-positive strains were VP7 genotyped, whereas three strains remained untypeable for the G type. Among the typeable strains, G1 (39.6 %) viral strains were found to be predominant followed by G3 (22.2 %), G4 (13 %), G9 (11.5 %), G2 (5.2 %) and G12 (5.2 %). Six samples (2.2 %) were genotyped as mixed infections: five cases of G1/G9 co-infections and one case of G1/G3 (Table 1).

Moreover, comparison between PAGE and G-typing results allowed us to observe that strains with G2 VP7 specificity presented with short electropherotypes, whereas strains with non-G2 VP7 specificity showed long profiles.

**P-typing results**

A total of 260 strains (96.3 %) were successfully VP4 genotyped, whereas 10 strains remained P untypeable. Among the typeable strains, three VP4 genotypes co-circulated in Tunisia between 2009 and 2014. P[8] (74.5 %) was the predominant P genotype. P[6] (10.4 %) and P[4] (5.5 %) were also quite commonly detected. Mixed VP4 types were noted in 16 cases (6.1 %) (Table 1).

**G/P-genotype combinations**

A total of 257 strains (95.2 %) were successfully G- and P-genotyped. Twenty-two different G/P combinations were found to co-circulate in Tunisia between 2009 and 2014. The most common genotype combinations were G1P[8], G3P[8], G9P[8], G4P[8], G2P[4] and G12P[6]. Depending on the year, mixed genotypes and unusual combinations such as G1P[4], G2P[6], G2P[8], G3P[4], G3P[6], G4P[6], G9P[6] and G12P[8] were also detected (Table 1).

**DISCUSSION**

This study extends previous epidemiological surveillance on RVA disease and genotype prevalence in Tunisian children <5 years of age consulting for acute gastroenteritis since 1995 (Chouikha et al., 2007, 2011a; Trabelsi et al., 2000, 2010), and this prior to the introduction of RVA vaccination in the national childhood vaccination programme.

**RVA detection rate**

During the present study, RVA was found in 270 (24 %) of the 1127 stool samples examined. This rate is similar to those reported in the Tunisian studies conducted between 1995 and 2008 by the national RV surveillance group (17.3–26.2 %) (Ben Hadj Fredj et al., 2012; Chouikha et al., 2009, 2011a; Trabelsi et al., 2000, 2010). Moreover, it is comparable to the detection rates reported for African countries where RV vaccine is not yet included in the free national childhood programme: 25.2 % in Egypt (Matson et al., 2010), 18 % in Tanzania (Moyo et al., 2007) and 18 % in Nigeria (Aminu et al., 2010). However, the prevalence of RVA infection in the present study is lower than those found in Morocco (42 %) (Benhafid et al., 2013), Cameroon (42.8 %) (Ndze et al., 2012), southern Ghana (48.2 %) (Enweronu-Laryea et al., 2013), south-eastern Nigeria (56 %) (Tagbo et al., 2014) and India (35.4 %)
Such differences may be partly due to the inclusion criteria of each study; differences in the period of study (sampling done during the whole year or focused on only during the outbreak period) and differences in the study populations (children <5 years old versus children <2 years old or sometimes patients of all ages; hospitalized children versus outpatients). Although the RVA detection rate varies from one study to another and from one region to another, in geographic regions where vaccination is not yet systematically provided, RVA remains a virus responsible for a high level of morbidity, and these data are true for both developed and developing countries; hence, its qualification as a ‘democratic’ virus (Glass et al., 1999).

In the present study, children hospitalized for diarrhoea were more likely to have RVA detected (24.9 %) than those consulting as outpatients (18.5 %), confirming that RVA gastroenteritis is usually more severe than non-RVA diarrhoea (El-Shabrawi et al., 2015; Malek et al., 2010).

The present study, associated with previous reports of RVA infection in Tunisia (Hassine-Zafrane et al., 2011; Trabelsi et al., 2000, 2010), allowed observation of a seasonal pattern of RVA infection characterized by marked outbreak peaks occurring during the coolest months of the years; RVA was found to occur predominately in autumn and winter, with peaks observed annually between November and March. This seasonality is typical of that seen in many temperate climates.

Table 1. VP7 and VP4 genotypes of RVA strains isolated in Tunisia between 2009 and 2014

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<tbody>
<tr>
<td>G1</td>
<td>1 (0.4)</td>
<td>5 (1.9)</td>
<td>93 (34.4)</td>
<td>5 (1.9)</td>
<td>3</td>
<td>107 (39.6)</td>
</tr>
<tr>
<td>G2</td>
<td>11 (4)</td>
<td>1 (0.4)</td>
<td>1 (0.4)</td>
<td>1 (0.4)</td>
<td>0</td>
<td>14 (5.2)</td>
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<tr>
<td>G3</td>
<td>2 (0.7)</td>
<td>6 (2.2)</td>
<td>1 (0.4)</td>
<td>11 (4.1)</td>
<td>1</td>
<td>60 (22.2)</td>
</tr>
<tr>
<td>G4</td>
<td>1 (0.4)</td>
<td>5 (1.9)</td>
<td>1 (0.4)</td>
<td>1 (0.4)</td>
<td>0</td>
<td>16 (6)</td>
</tr>
<tr>
<td>G9</td>
<td>0</td>
<td>3 (1.1)</td>
<td>28 (10.3)</td>
<td>0</td>
<td>0</td>
<td>31 (11.5)</td>
</tr>
<tr>
<td>G12</td>
<td>0</td>
<td>7 (2.6)</td>
<td>1 (0.4)</td>
<td>1 (0.4)</td>
<td>0</td>
<td>14 (5.2)</td>
</tr>
<tr>
<td>G mixed</td>
<td>0</td>
<td>0</td>
<td>5 (1.9)</td>
<td>0</td>
<td>0</td>
<td>6 (2.2)</td>
</tr>
<tr>
<td>G untyped</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>3 (1.1)</td>
</tr>
<tr>
<td>Total, No. (%)</td>
<td>15 (5.5)</td>
<td>28 (10.4)</td>
<td>201 (74.5)</td>
<td>16 (5.9)</td>
<td>10 (3.7)</td>
<td>270 (100)</td>
</tr>
</tbody>
</table>
In the present study, nine different electropherotypes were detected between 2009 and 2014 in the coastal region of Tunisia. In general, each RVA strain showed a dsRNA migration pattern (electropherotype) on polyacrylamide gels distinct from that of other strains. Analysis of such genomic polymorphism as determined by PAGE has been routinely used to assign the parental gene origin of a reassortant. In our study, PAGE was seen to be relatively insensitive. We detected only 182 (67.4%) of 270 confirmed RVA-positive stool specimens by this method. Various factors and running conditions of PAGE have been reported to affect the electrophoretic pattern of each strain, including the concentration of acrylamide/bisacrylamide, type of running buffer, magnitude of running voltage and purity of dsRNA analyzed (Chouikha et al., 2011b; Saravanan et al., 2004).

Concerning the relationship between electropherotypes and G/P combinations, results are in agreement with those previously reported. Indeed, analysis of electropherotypes distribution according to VP7 specificities showed that some genotypes were frequently associated with a particular migration pattern. Thus, we observed that G2 strains were exclusively found in conjunction with short electropherotypes, while non-G2 strains always exhibited long electropherotypes (Chouikha et al., 2011b; Saravanan et al., 2004).

**G/P-typing**

Of the 270 strains analyzed, 257 (95.2%) could be both G- and P-genotyped, whereas 13 (4.8%) could be genotyped only for G or P specificity. The notion of untypeable strains, mentioned in several studies, seems to result from several possible factors including the emergence of new genotypes with unknown specific primers, as well as point mutations in hybridization regions of the primers. Therefore, one of the proposed solutions is the use of degenerate primers, but the most effective typing method remains sequencing following amplification of the entire gene (Iturriza-Gomara et al., 1999).

During the study period (2009–2014), we identified the widespread circulation of G1 and G3 genotypes (39.6% and 22.2%, respectively), with limited circulation of G4 (13%), G9 (11.5%), G2 (5.2%) and G12 (3.2%). As expected, and compared to previous Tunisian studies, the distribution of RVA strains varied considerably. During a 10-year period (1995–2004), G1 was the most predominant genotype (59%) in Tunisia, followed by G4 (10%), G3 (9%), G2 (2%) and G9 (1%) (Chouikha et al., 2007).

In the present study, the G9 genotype was detected with a prevalence of 11.5%. The G9 strain was first detected in the USA in 1983–1984. Its prevalence increased markedly around the world in the late 1990s (Santos & Hoshino, 2005). In Tunisia, the G9 genotype was isolated for the first time in 2004 and its rate detection remained quite low (2.5–3.3%) until 2007 (Chouikha et al., 2009).

In this study we report, for the first time in Tunisia, the multiple detection of an emerging human RVA strain, the G12 genotype. In fact, G12 RVA strains may have been circulating in Tunisia prior to this study but their detection was not achieved by previously used RT-PCR genotyping, as the G12-specific primer was included in the PCR mixture. The first detection of a G12 genotype was performed by sequencing of the ninth gene segment of the VP7 untypeable strain (Ben Hadj Fredj et al., 2013a). Such a finding allowed us to further optimize an RT-PCR genotyping protocol that is able to detect the G12 strains. This genotype appears to have spread more rapidly in recent years. It was isolated for the first time in the Philippines in 1987 (Taniguchi et al., 1990) and was further reported in 1998 in Thailand (Pongsuwanna et al., 2002). Subsequently, G12 RVA has spread rapidly all over the world and is currently considered the sixth most important global genotype (Matthijnssens et al., 2009; Rahman et al., 2007). In the present study the G12 genotype, with a rate detection of 5.2%, corresponded to the fifth genotype behind G1, G3, G4 and G9. This rate is comparable to that found in Bangladesh (5.6%) (Rahman et al., 2007), Saudi Arabia (4%) (Kheyami et al., 2008), Hungary (6.9%) (Bánya et al., 2007), Malawi (5%) (Cunliffe et al., 2009) and Manipur in Northeast India (8%) (Mukherjee et al., 2010).

During the study period, P[8], P[4] and P[6] co-circulated in Tunisia. While genotype P[8] predominated with a rate detection of 74.5%, the genotype P[4] was detected only at a rate of 5.5%, leaving genotype P[6] as the second most detected with a rate of 10.4%. This result is comparable to those found in previous Tunisian studies conducted between 1995 and 2004 (Chouikha et al., 2007; Trabelsi et al., 2010).

According to the literature, although P[4] and P[6] are predominant in countries such as Brazil for P[4] (Soares et al., 2012) and Nigeria for P[6] (Aminu et al., 2010), P[8] is known to represent the most frequent VP4 genotype worldwide (Cunliffe et al., 2009; Esse et al., 2010; Le et al., 2008; Uchida et al., 2006).

Globally, at least 73 G/P-genotype combinations of RVAs infecting humans have been described (Matthijnssens et al., 2009). The RVA strains G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and, to a lesser extent, G12P[8] currently represent the most frequent genotype combinations in humans worldwide (Matthijnssens & Van Ranst, 2012).

In the present study, 22 distinct associations were identified. G1P[8] represents the predominant combination circulating in Tunisia, with a prevalence of 34.4%. Between 1995 and 2004, G1P[8] was the predominant RVA strain in Tunisia (Chouikha et al., 2007; Trabelsi et al., 2000, 2010). Between 2005 and 2008, this association periodically gave way to G3P[8] (in 2005/2006) or G2P[4] strains (in 2007) (Ben Hadj Fredj et al., 2012; Chouikha et al., 2011a).

In this study, G12 VP7 specificity was found in association with P[8] (five cases (1.9%)) and P[6] (seven cases...
In African countries G12 strains were first detected in South Africa, mostly in association with P[6] (Page et al., 2009). G12P[8] strains recently emerged as a predominant strain in western Africa (Ndze et al., 2013; Oluwatoyin Japhet et al., 2012). According to published data, although G12 genotypes have already been reported in association with VP4 genotypes P[4], P[7] and P[9], the most common associations found worldwide remain those with genotypes P[8] and P[6] (more than 90%) (Matthijnssens et al., 2010).

Most associations found in the present study correspond to stable combinations involving genotypes with strong affinities. Indeed, the genotype P[4] is commonly linked to the G2 genotype, while the genotype P[8] is often associated with genotypes G1, G3, G4, G9 and G12 (Matthijnssens & Van Ranst, 2012). However, this molecular study allowed us to highlight the circulation of RVA strains with uncommon G/P-genotype combinations in 9.4% of cases, such as G1P[4], G1P[6], G2P[6], G2P[8], G3P[4], G3P[6], G4P[4], G4P[6] and G9P[6]. The existence of such associations has previously been reported in other studies (Argüelles et al., 2000; Ben Hadj Fredj et al., 2012; Mukherjee et al., 2010). This reflects the great variability of RVA and the possibility of reassortment among strains.

In the present study, the rate of detection of mixed infections with G/P-genotyping was 7.8%. The occurrence of mixed infections appears to be more common in developing countries where, in general, a broad genotype diversity can be seen (Soares et al., 2012). Such co-infections can conducive to the emergence of unusual G/P combinations that can sporadically acquire relevance in certain geographical settings and populations. Moreover, possible reasons for the higher diversity observed in Africa and Asia include housing in close proximity to domestic animals from which the shedding of virus occurs (Sharma et al., 2009).

Rearrangement, reassortment, interspecies transmission and positive accumulation of point mutations represent the main forces driving the evolution of rotaviruses, and are responsible for the production of a heterogeneous population of viruses in continuous evolution (Papp et al., 2013).

Considerable diversity of RVA genotypes was present among strains circulating in Tunisia between 2009 and 2014. Unusual or emerging RVA strains are increasingly being reported and already constitute the most common strains in some areas. This is the first report of multiple detection of the G12 genotype in Tunisia. This study highlights the need for maintaining active surveillance of emerging RVA strains in northern Africa and timely recognition of novel or rare strains with unusual G/P type combinations. Such studies allow monitoring of changes in RVA disease burden and strain dynamics and the detection changes over time. Moreover, our result can help health officials monitor the impact of vaccination programmes on genotypic prevalence, and provide information on the ability of vaccination to protect against RVA diarrhoea. The greater genetic diversity described in developing countries might be attributable to the close relationship between humans and livestock in daily life in certain communities, favoring the emergence of new RVA strains. Rearrangement, reassortment, interspecies transmission and positive accumulation of point mutations represent the main forces for this diversity. Continued surveillance of RVA strains will provide more insights into interspecies transmission processes of RVAs. In turn, this information could help determine how the introduction of novel genes might affect the evolution of the RVA populations that infect humans.

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