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Molecular characterization of rotaviruses circulating in the population in Turkey

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SUMMARY

Of a total of 508 stool specimens from children with acute diarrhoea, mostly under the age of 5 years, collected in nine cities in the western and southeastern regions of Turkey between May 2000 and October 2002, 119 (23·4%) were found positive for rotaviruses (RV) by ELISA. Positive samples were characterized by electropherotyping and G and P genotyping. A subset of G and P types were confirmed by nucleic acid sequencing. The most prevalent types found in this collection included G4P[8], accounting for 27/64 (42·2%) of the fully characterized strains. G1P[8], G2P[4] and G3P[8] were found in 17 (26·6%), 2 (3·1%) and one (1·5%) samples respectively. Less common strains such as G9P[8] were found in two (3·2%) samples and G2P[8], G1P[6], G2P[6] and G4P[6], possible reassortant viruses, were found in five (7·8%), 2 (3·1%), one (1·5%) and four (6·3%) samples respectively. Mixed infections were found in six (7·3%) samples and were associated with combinations of G1 + G2, G1 + G4, G1 + G9 and G4 + G9 strains. This is the first molecular epidemiology study of its kind to be carried out in Turkey and suggests a significant diversity of co-circulating rotavirus strains.

INTRODUCTION

Human rotavirus (HRV) is the primary aetiological agent of gastroenteritis in infants worldwide [1]. In developing countries, it is estimated that rotavirus is responsible for one third of all diarrhoea-associated hospitalizations and up to 600000 deaths annually [2, 3]. In industrialized countries, where mortality is low, nearly all children experience an episode of rotavirus diarrhoea in the first 5 years of their lives [4–6].

A dual typing system classifies HRVs by VP7 serotype (or G type, since VP7 is a glycoprotein) and by VP4 serotype (or P type, since VP4 is a protease-sensitive protein) [7]. To overcome this difficulty, group A rotavirus strains are classified into G and P types [8–10]. The incidence of infection with particular group A rotavirus genotypes varies between geographical areas during a rotavirus season and from one season to the next [11–13]. Globally, viruses carrying either G1, G2, G3, or G4 and P[4] or P[8] are the most common causes of rotavirus disease in humans [14–20]. Surveys indicate that G9 strains have been found worldwide since the mid-1990s [21], G5 strains in Brazil, and G8 and G10 strains in Brazil [15–17], India [18, 19] and Africa [20]. The routine implementation of safe and effective rotavirus vaccine programmes in developing countries is expected to dramatically reduce the high level of mortality attributed to HRV, but the differing epidemiology of rotavirus infection in such settings may pose special challenges to successful vaccine use [21, 22]. In particular, the greater diversity of HRV strains

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encountered in developing countries suggests that before formulation of rotavirus vaccines, global surveillance and detailed characterization of the strains in circulation should be undertaken in order to incorporate other serotypes for a future candidate rotavirus vaccine.

Thus, it is obvious that a global screening programme is needed to broaden our knowledge about the molecular characteristics of this virus. To extend these observations and introduce rotavirus characterization a pilot collection of 119 Turkish rotavirus strains was examined. The distribution of G and P types was analysed by molecular methods, including polyacrylamide gel electrophoresis (PAGE), RT–PCR, and nucleic acid sequencing. Common types found worldwide were the predominant genotypes. Some uncommon types and mixed infections were also found suggesting that a complex pool of rotavirus strains co-circulate in each geographical location which may make the effort to develop an effective vaccine candidate more difficult.

MATERIALS AND METHODS

Viruses

A total of 119 stool samples collected in three consecutive (April to late October) seasons from 2000 to 2002 were used in this study. All submitted samples were taken from children (age range 16 days to 7 years, median 9 months) suffering acute diarrhoea of unknown viral aetiology. Samples were obtained from nine cities located in four different geographical regions in the western, southern, and eastern parts of Turkey. Stool samples were stored at ambient temperatures (35–45 °C during the spring–summer seasons) as there was no cold room available in any of the local hospitals. The samples were identified as rotavirus-positive by antigen detection strategies, including enzyme immunoassays (Dako kit, Glostrup, Denmark) and latex agglutination tests (Orion Diagnostics, Espoo, Finland). Faecal samples were stored on receipt in Gaziantep at −40 °C prior to rotavirus characterization. Reference strains, ST3, Ds5, and Wa, were kindly provided by Dr Ruth Bishop. Sequences were deposited in the rotavirus sequence database held at the Enteric Virus Unit (Centre for Infections, Health Protection Agency, London, UK) and are freely available upon request to the authors (miren.iturriza@hpa.org.uk).

RNA extraction and PAGE

The double-stranded RNA segmented genome characteristic of rotavirus was isolated from samples using a standard phenol–chloroform extraction method with ethanol precipitation [23]. RNA was treated with DNase1 (Roche Molecular Biochemicals, East Sussex, UK) to remove contaminating genomic DNA. Electropherotypes of strains were determined using a 10% polyacrylamide gel with a discontinuous buffer system [24] followed by silver staining using the method of Herring et al. [25].

Rotavirus genotyping

Nucleic acid extraction and reverse transcription were performed as previously described [23]. To determine the G and P types, RT–PCR assays were performed. Initially, an 881-bp product from segment 9, encoding the VP7 glycoprotein in human group A rotaviruses, was amplified using primer VP7-F (5′-ATG TAT GGT ATT GAA TAT ACC AC-3′) and primer VP7-R (5′-AAC TTG CCA CCA TTT TTT-3′). This was followed by multiplex hemi-nested PCR using the genotype-specific primers which identify G types [26]. To identify P genotypes, a DNA fragment of 876-bp, from gene segment 4 (encoding VP4), was amplified using the consensus primers Con3 (5′-TGG CTT CGC CAT TTT ATA GAC A-3′) and Con2 (5′-ATT TCG GAC CAT TTA TAA CC-3′), followed by genotyping with P-typing primers [27]. Amplified products from the RT–PCR assays were analysed in conventional 2% agarose gels stained with ethidium bromide (0·1 mg/ml) and visualized using a UV transilluminator.

Nucleic acid sequencing

A subset of DNA fragments was chosen to validate the G- and P-typing methods and first round PCR amplicons which did not genotype in the second round of the PCR were sequenced in order to characterize these strains. Also, the NSP4 genotype was determined for a small number of strains in order to identify the species of origin of the strains. The first round PCR products of both P- and G-typing reactions or the amplicon derived from an NSP4-specific RT–PCR were purified (Geneclean, Qbiogene, Cambridge, UK), and sequenced using dye terminator chemistry protocols with cycle sequencing (Beckman Instruments, Inc., Fullerton CA, USA). The sequences

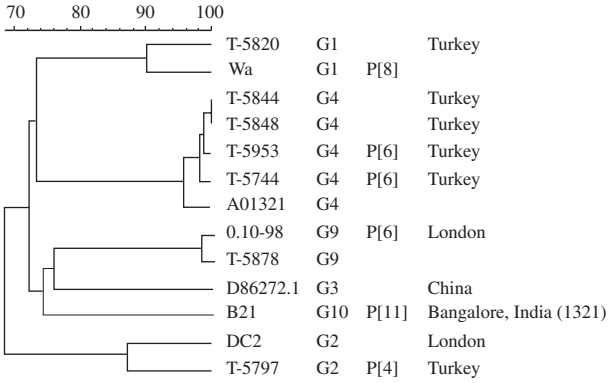

Fig. 1. Dendrogram (maximum parsimony) of partial sequences (nt 51–932) of the gene encoding human rotavirus VP7. Accession numbers of sequences obtained from GenBank and included in the comparisons are indicated in the figure. Other sequences used in comparison, including those from the strains isolated in Turkey are deposited in the database held at the Enteric Virus Unit, Health Protection Agency, London, UK.

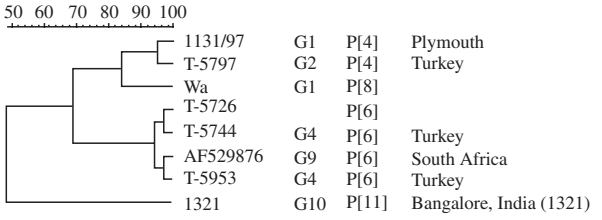

Fig. 2. Dendrogram (maximum parsimony) of sequences corresponding to the VP8* region of the gene encoding human rotavirus VP4. Accession numbers of sequences obtained from GenBank and included in the comparisons are indicated in the figure. Other sequences used in comparison, including those from the strains isolated in Turkey are deposited in the database held at the Enteric Virus Unit, Health Protection Agency, London, UK.

were compared against sequences in a database containing a collection of sequences of G, P and NSP4 encoding genes of over 400 isolates of human and animal rotaviruses. Data analysis was performed using Clustal for multiple alignments and neighbour joining for phylogenetic analysis (Bionumerics, Kortrij, Belgium).

RESULTS

Determination of electropherotypes

The arrangement of 11 RNA segments obtained from isolates by electrophoretic separation revealed a general migration pattern 4–2–3–2 similar to that found in the reference group RV5 and ST3 (data not included). Most of the rotavirus strains detected by PAGE showed the long electrophoretic pattern with the exception of one strain which exhibited a short electropherotype. Electrophoretic patterns of isolates are slightly different from the reference strains (RV5 and ST3). Electrophoretic patterns of some isolates were even different from each other. Thus, variation among strains is evident.

Table 1. *The distribution of rotavirus G and P types in Turkey between 2000 and 2002*

	P[4]	P[6]	P[8]	n.d.	Total
G1		2	17	7	26
G2	2	1	5		8
G3			1		1
G4		4	27	6	37
G9			2	2	4
G1 + G2			1		1
G1 + G4			2	1	3
G1 + G9				1	1
G4 + G9				1	1
n.d.		1	8		9
Total	2	8	63	18	91

n.d., Not determined.

Determination of G and P types

Out of 119 isolates tested, 91 were G- and/or P-typed. Sixty-four were both G- and P-typed, 18 were G-typed only and nine were P-typed only. Of the total collection number (119), 28 were found to be untypable although initial EIA tests and an RT–PCR to amplify a region of the VP6 gene were both positive. Partial DNA sequencing of the first round amplicons of VP7 and VP4 encoding genes of representative G and P types confirmed the genotypes identified by hemi-nested PCR. Also, the sequencing of amplicons from PCR reactions in which a genotype was not identified allowed the genotype to be identified through phylogenetic analysis (Figs 1 and 2).

The most prevalent G- and P-type combination found was G4P[8], accounting for 27/64 (42·2%) of the fully characterized strains. G1P[8], G2P[4] and G3P[8] were found in 17 (26·6%), two (3·1%) and one (1·5%) samples respectively (Table 1). Less common strains such as G9P[8] were found in two (3·2%) samples and G2P[8], G1P[6], G2P[6] and G4P[6], possible reassortant viruses, were found in five (7·8%), two (3·1%), one (1·5%) and four (6·3%) samples respectively (Table 1). Mixed infections were found in six (7·3%) samples and were associated with

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Table 2. *The seasonal distribution of rotavirus G and P types in Turkey between 2000 and 2002*

	P[4]	P[6]	P[8]	n.d.	Total
2000					
G1			4	1	5
G2	1	1	1		3
G3					
G4		2	2		4
G9				1	1
G1+G2			1		1
G1+G4			2		2
G1+G9					
n.d.		1	6		7
Total	1	4	16	2	23
2001					
G1		2	5	6	13
G2	1		4		5
G3					
G4		2	10		12
G9			1	1	2
G1+G2					
G1+G4				1	1
G1+G9					
n.d.			2		2
Total	1	4	22	8	35
2002					
G1			8		8
G2					
G3			1		1
G4			15	6	21
G9			1		1
G1+G2					
G1+G4					
G1+G9				1	1
G4+G9				1	1
n.d.					
Total			25	8	33

n.d., Not determined.

combinations of G1+G2, G1+G4, G1+G9 and G4+G9 strains (Table 1).

Seasonality

The distribution of rotavirus genotypes varied with each rotavirus season. G1P[8] was predominant in the first season (2000), whereas in 2001 and 2002, G4P[8] predominated (Table 2). Also, strains of P type, P[6] were found in the first two seasons but not in the third (Table 2). All the samples were obtained from children. The age range was between 16 days and 7 years (median 9 months). In the majority of the cases, the illness seemed to start with a severe diarrhoea and a temperature of 38·8 °C. Vomiting was rarely seen in infants with the illness.

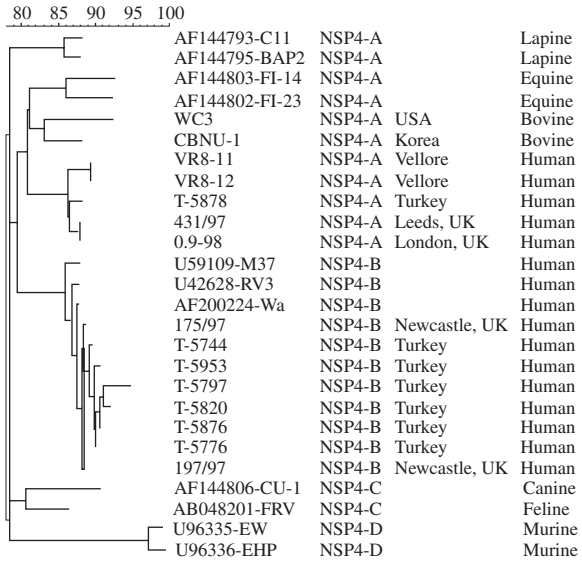

Fig. 3. Dendrogram (maximum parsimony) of sequences of the gene encoding rotavirus NSP4. Accession numbers of sequences obtained from GenBank and included in the comparisons are indicated in the figure. Other sequences used in comparison, including those from the strains isolated in Turkey are deposited in the database held at the Enteric Virus Unit, Health Protection Agency, London, UK Species of origin of the rotavirus strains is indicated in the far right column.

NSP4 A and B determination

Characterization of the gene encoding the non-structural protein NSP4 indicated that samples were of NSP4 genotypes A or B and clustered with HRV NSP4 lineages. Moreover, the majority of the NSP4 sequences were found to be of genotype B which is an indication of a long electropherotype. The sequencing of amplicons from PCR reactions allowed the NSP4 genotype to be identified through phylogenetic analysis (Fig. 3).

DISCUSSION

Rotavirus is the principal cause of infantile diarrhoea worldwide and has a significant impact on morbidity and mortality in both developing and industrialized countries. Antigenic and molecular analysis-based methods available for the characterization of rotaviruses have facilitated the identification of the predominant genotypes circulating globally, as well as the detection of uncommon, reassortant and zoonotic strains. The degree of diversity among rotavirus

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strains has been reported to be higher in some regions of the world than in others [14–20]. Uncommon human strains such as G5, G8, G9, and G10 were until recently found only in countries such as Brazil and India [15–19]. However, some of these strains, such as G9, are emerging as global strains [21]. This observation has implications for the development of a suitable rotavirus vaccine.

In this study, 119 Turkish rotavirus samples were investigated. Electrophoretic analyses of the samples showed the presence of rotavirus strains. G and/or P types were obtained for 91 strains. The failure to genotype some of the strains may have been associated with suboptimal storage conditions in which the faecal samples were kept for some time between 35 °C and 45 °C during collection and transportation which might cause degradation of capsid proteins and RNAs.

Analysis of the genotyping data indicated that both common and uncommon genotypes were co-circulating in Turkey between 2000 and 2002 (Tables 1 and 2). Among these, G4P[8] and G1P[8] were the most common genotypes found, consistent with the majority of studies worldwide. Possible reassortant strains such as G1P[6], G2P[6], G2P[8] and G4P[6] were not uncommon in this study. In particular, the incidence of infection with G types in combination with P[6] may suggest the zoonotic transmission, in the near or distant past, of common animal rotavirus strains, many of which occur in combination with P[6]. G9P[8] strains which are thought to be a reassortant between a G9 animal rotavirus strain and a common P[8] human strain and which have been increasingly recognized worldwide were also found in Turkey in all three years of the study. Mixed infections, which are a prerequisite for reassortment, were found in all three years but were more prevalent in 2000 when the diversity of co-circulating rotavirus strains was highest. Electropherotyping of the strains showed that a high level of strain diversity was apparent. The majority of G4P[8] genotypes had long electropherotypes. The same was also true for G4P[6] (one short electropherotype). Interestingly, these short electropherotypes were from different western cities. Even though G4P[8] strains in Turkey still seemed to be the predominant and stable type for the past 3 years (Tables 1 and 2), the difference in electropherotypes prove that temporal and geographical variances from city to city were evident. This underscores the need for a comprehensive vaccine that will provide immunity despite the change in genotype depending on the geographical distance.

It seems that the illness is mainly associated with infants rather than children >5 years of age or teenagers. This is especially true for families living in arid zones with their animals where there is a year round sub-Saharan climate and far from the sea. The illness spreads rapidly in large families with more than five children, but the severity of the illness is less in these families. This could be due to the presence of protective antibodies in their mothers.

Characterization of the gene encoding the non-structural protein NSP4 indicated that all strains detected were of human origin although the presence of unusual G and P genotype combinations may suggest zoonotic transmission in the past and their subsequent reassortment with common co-circulating human strains. The majority of the strains tested were of NSP4 genotype B which is consistent with strains of longer electropherotypes. This was the case in PAGE analyses of strains from different samples.

As reported in other studies, mixed infections were frequent in years in which several diverse rotavirus strains were co-circulating in the population [11] and are a prerequisite for reassortment.

Given that present rotavirus vaccines are aimed at eliciting serotype-specific immunity to RV, our results indicate that a successful vaccine against rotavirus in Turkey should elicit immunity or cross-protection to G4, G1, G2, G3, and G9 also. Whether the presently licensed vaccine candidates can elicit protective immunity to each serotype of G4, G1, G2, G3, and G9 remains to be demonstrated conclusively. Thus, this study reinforces the need to conduct ongoing molecular epidemiological studies of co-circulating rotavirus strains. The successful introduction of any vaccination programme may be dependent on the diversity of co-circulating rotavirus stains and the dynamic nature of this diversity.

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