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Detection of human rotavirus serotype G6 in Hungary

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SUMMARY

During an ongoing survey of human rotavirus serotypes, we demonstrated for the first time the circulation of serotype G6 in two regions of Hungary. Of five rotavirus seasons surveyed to date (1994–9), serotype G6 was found in all seasons except 1994–5 at an overall prevalence of 1·4% (17 of 1252) and ranging from 0·6 to 4·5%. Children infected with G6 strains were older (mean age, 3·3 years) than children infected with the four (G1–G4) globally common serotypes (mean age, 2·1 years; unpaired Student's *t* test, $P<0{\cdot}001$). Our data indicate that rotavirus serotype G6 may be an epidemiologically important G serotype in Hungary.

INTRODUCTION

Group A rotaviruses are the most common cause of severe dehydrating diarrhoea in childhood [1]. Improvements in sanitation have little effect on control of the disease; thus, active immunization appears to offer the best strategy to protect against infections and to decrease the disease burden associated with rotavirus [2]. A tetravalent reassortant vaccine (rhesus rotavirus tetravalent vaccine, RRV-TV) with serotype specificities of the 4 globally common serotypes (G1–G4) was developed in part because of the perception that homotypic (serotype-specific) protection was important for immunity [3, 4]. In field trials conducted in both developing and industrialized countries, vaccine efficacy was $\sim 50\%$ for the prevention of any rotavirus disease and $>80\%$ against severe diarrhoea [5, 6]. RRV-TV (RotaShield™, Wyeth Laboratories Inc., PA, USA) was licensed in 1998 by the US Food and Drug Administration and used for 9 months in the United States before it was withdrawn due to a rare association with intussusception in vaccinees [7–9]. However, other experimental monovalent and multivalent vaccines are being developed [10, 11].

To plan for possible vaccination programmes, many countries have initiated or are planning formal surveillance programmes to better understand the rotavirus disease burden and the serotype diversity of strains, and to subsequently study the impact of vaccines on serotype prevalence, and to help determine the degree to which vaccines may provide heterotypic protection, once vaccination programmes have been initiated. Rotavirus surveillance with application of reverse transcription polymerase chain reaction (RT–PCR) to characterize strains has in many parts of the world led to the increased detection of uncommon rotavirus strains (non-G1–G4). Some of these uncommon strains have a regional distribution (e.g. serotype G5 in Brazil, G8 in Malawi and parts of Africa), but at least one serotype previously considered to be rare, G9, circulates globally and has emerged to become 1 of the 4 or 5 most prevalent strains worldwide [12–17]. Rotaviruses with G6 specificity are recognized as a common serotype in cattle but are uncommon in humans, and only a few isolated cases have been identified in association

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with human infections by Italian, Australian, Indian, and US investigators [18–23].

We recently set up a surveillance system in two regions of Hungary to help define the strain diversity of rotavirus in anticipation of a possible vaccination programme. As part of this surveillance, we have identified the first human serotype G6 strains in Hungary and found that they have been in circulation for at least 4 years.

METHODS

Stool specimens for this study were obtained between 1994 and 1999 from hospitals in two areas of Hungary, 150 km apart. Routine strain characterization was performed by RNA profile analysis (electropherotyping) using polyacrylamide gel electrophoresis and silver staining (PAGE), and serotype-specific monoclonal antibody enzyme immunoassay (MAb-EIA) [24–27]. Strains were serotyped initially by MAb-EIA with MAbs specific for the common serotypes, G1–G4 [25–29].

We attempted to genotype 58 strains for which a serotype could not be determined with MAb-EIA by multiplex RT–PCR, using a primer set developed to genotype human rotaviruses with G1–G4, and G9 specificity [30].

Those specimens reactive with the type-common MAb but non-reactive with type-specific G1–G4 MAbs and also non-typable by RT–PCR were further tested by MAb-EIA serotyping assay with MAbs specific for unusual serotypes G5, G6, G9, and G10 [18, 31, 32].

To confirm our serotyping results, we determined the nucleic acid sequence of the VP7 gene for 6 strains representing the 3 major electropherotypes associated with G6 specificity, including 3 G6 MAb-reactive specimens. A distance matrix was generated comparing Hungarian G6 strain Hun8 (GenBank accession no.: AJ488135) to representatives of the 15 rotavirus G serotypes described. The *P*-distance values were estimated on the basis of the partial VP7 gene coding sequence (corresponding to nt 148–819 and aa 34–257) using the algorithm supplied in the phylogenetic software package MEGA2 [33].

To assign the type specificity three techniques, MAb-EIA serotyping, multiplex RT–PCR genotyping with primers specific for Hungarian G6 strains, and nucleic acid sequencing [18, 22, 26, 27, 30, 34] were applied; a single method was used to assign the G type specificity for 8 samples (MAb-EIA for 1 sample; RT–PCR for 7 samples) and 2 or all 3 methods were utilized for 9 samples, including 3 specimens each by MAb-EIA and RT–PCR, sequencing and RT–PCR, and MAb-EIA and sequencing and RT–PCR.

RESULTS

Among 1252 specimens characterized by MAb-EIA in the surveillance study, 79% were of serotype G1–G4 (G1, 61·5%; G2, 15%; G3, 1·5%; G4, 1%), 2·4% were mixed infection, and 18·6% were non-typable. Using the RT–PCR genotyping procedure, we genotyped an additional 42 strains (26 G1, 11 G2, 4 G4 strains and 1 G9 strain).

Thus, overall we were able to G type 85·4% of the samples (G1, 64%; G2, 16%; G3, 1·5%; G4, 1·5%; G9, $<0{\cdot}1\%$, $n=1$; dual infection, 2·4%), whereas 14·6% of the strains remained non-typable. Most of the non-typable specimens showed RNA profile identity with those samples for which a serotype specificity (G1–G4) could be assigned. To date, these strains have not been further analysed. However, we found 17 untypable samples that had several different RNA profiles that were also distinct from those characteristic of G1–G4 strains.

On the basis of exclusive reactivity with the G6-specific MAb-IC3 [18], 7 specimens were identified as serotype G6 (Table 1). These 7 samples represented 3 major RNA profiles (arbitrarily designated as A, B, and C). All 3 electropherotypes displayed a long RNA profile. RNA profiles A and C exhibited similar patterns, with slight differences in the migration of segments 4, 7, 8, 9, and 10, while profile B was unique and included only one specimen (Fig. 1).

Sequence data confirmed that all six strains selected for sequence analysis belonged to serotype G6 as shown by high homology with G6 strains and particularly with human G6 strains (Table 2). The homology of Hun8 VP7 with the VP7s of other Hungarian G6 strains representing RNA profiles A and B was 86 and 80% for the nucleic acid sequences, and 94 and 92% for the deduced amino acid sequences, respectively (data not shown). A complete molecular characterization of these G6 strains and a description of RT–PCR primers to detect them is in progress (Bányai et al., unpublished results).

Overall, 1·4% (17/1252) of the samples were identified as serotype G6. Five (2·4%), 2 (0·7%), 1 (0·6%), and 6 (3·4%) isolates with G6 specificity were identified in Budapest during 1995–6, 1996–7, 1997–8, and 1998–9, respectively, and 3 (4·5%) samples representing serotype G6 were found in Baranya County during

Table 1. *MAb-EIA reactivity pattern and RNA profile of stool samples designated as serotype G6, collected in Budapest and Baranya County, Hungary*

Sample	Absorbance values with the indicated MAbs*			RNA profile†
	MAb/60 (common VP7)	G6-IC3	Others‡	
1	1·79	1·46	⩽0·1	A
2	1·19	1·01	⩽0·1	A
3	0·86	1·58	⩽0·1	B
4	1·32	0·72	⩽0·1	C
5	1·34	0·3	⩽0·1	C
6	1·84	0·85	⩽0·1	C
7	1·73	1·79	⩽0·1	C
G6 control (PA169)	1·67	1·84	⩽0·1	ND§
Negative control (PBS)	<0·1	<0·1	<0·1	—

* Serotype-specific reactions defined by the homologous MAb had at least a twofold higher absorbance value with than those defined the heterologous MAb [28].
† RNA profiles were designated arbitrarily as shown in Figure 1.
‡ G type-specific MAbs were: G1-5E8, G2-1C10, G3-159, G4-2G7, G5-5B8, G9-F45:8, G10-B223N7 [18, 27–29, 31, 32].
§ ND; not determined in this experiment.

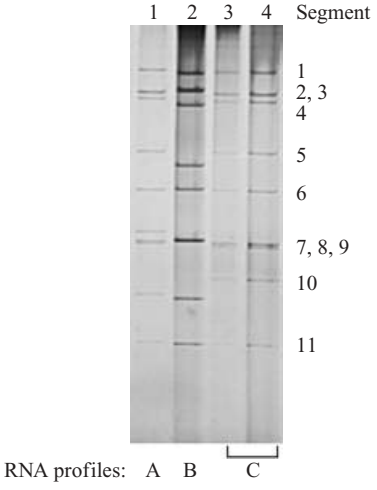

Fig. 1. The major RNA profiles of samples associated with G6 specificity. The RNA patterns, arbitrarily designated A and C, display some differences in the mobility of gene segments 4, 7, 8, 9, and 10. The arbitrarily designated B type RNA profile exhibits a different electrophoretic pattern. The samples in lanes 3 and 4 have the same RNA profile, although they were collected from different geographic areas, Budapest and Baranya County, respectively.

the 1997–8 season. No G6 strains were identified during 1994–5. Electropherotyping and nucleotide sequencing confirmed that distinct G6 strains circulated in different months of the same season and from season-to-season in both geographic areas (Table 3). Five strains exhibiting electropherotype A were detected in stools of children hospitalized in Budapest and circulated for at least 4 months during the 1995–6 season, and one additional sample with this electropherotype was identified in the middle of the following season. Only one sample representing the B-type RNA pattern was found, and it was collected from a child admitted to a Budapest hospital in March 1997. Electropherotype C showed a long-term circulation from December 1997 to February 1999 and was detected in Budapest and in Baranya County. Two additional samples, one each of electropherotypes A and C, were identified in January 1996 and December 1998, respectively, but because the quantity of stool sample was too small, we could not serotype or genotype them.

DISCUSSION

All children infected with serotype G6 were in-patients, suggesting the infections were relatively severe. Residence data were available only for specimens received from Baranya County. All 3 of these samples were obtained from patients who lived in rural settings. Patients infected with G6 strains were older (mean age, 3·3 years; median, 3·2 years; range, 0·8–6·4 years; $n_1 = 16$) than children infected with serotypes G1–G4 (mean, 2·1 years; median, 1·8 years; range, 0·1–13·5 years; $n_2 = 813$; unpaired Student's t test, $P < 0·001$).

Table 2. *Sequence homology for the outer capsid protein VP7 of a Hungarian rotavirus isolate* (*Hun8*) *displaying E-type 'C' to reference strains based on its partial nucleotide and amino-acid sequence* (*corresponding to nt 148–819 and aa 34–257*)

Strain (origin*)	GenBank accession no.	G serotype	Homology (%)	
			Nucleic acid	Amino acid
Wa (Hu)	K02033	1	70	77
KUN (Hu)	D50124	2	71	74
AU-1 (Hu)	D86271	3	74	84
Hochi (Hu)	AB012078	4	73	77
OSU (Po)	X04613	5	75	82
PA151 (Hu)	L20881	6	95	97
PA169 (Hu)	L20880	6	79	92
MG6 (Hu)	U22011	6	79	92
UK (Bo)	X00896	6	79	91
NCDV (Bo)	M12394	6	80	89
Ty-1 (Tu)	S58166	7	65	66
EGY1850 (Hu)	AF104102	8	73	83
US1205 (Hu)	AF060487	9	76	86
Mc35 (Hu)	D14033	10	73	82
YM (Po)	M23194	11	74	84
L26 (Hu)	M58290	12	72	79
L338 (Eq)	D13549	13	73	81
FI23 (Eq)	M61876	14	73	81
Hg18 (Bo)	AF237666	15	74	81

* Hu, human; Po, porcine; Bo, bovine; Tu, turkey; Eq, equine.

Table 3. *Seasonal and geographical distribution of human G6 rotaviruses in Hungary*

Source of samples	RNA profile*	1995	1996		1997			1998			1999	Total
		Dec.	Jan.	Mar.	Jan.	Mar.	Dec.	May	Nov.	Dec.	Feb.	($n=17$)
Budapest	A	3	1	1	1							6
	B					1						1
	C						1		1	3	2	
Baranya county	C						2	1				10

* Arbitrarily selected designations for distinguishing the different electropherotypes associated with G6 specificity (see Figure 1).

Infection with G6 strains in older children suggested lack of cross-protection from past rotavirus infection. Since second infections with common strains are typically milder, these symptomatic infections in older children suggest that G6 strains may represent new infections in the community and that G6-associated illness is not prevented by prior infection with the common strains [36]. If this hypothesis is true, it raises questions on whether G6 strains will emerge to cause large epidemics or differ significantly in their clinical manifestations or epidemiological features compared with disease caused by common rotavirus serotypes. We were unable to address these questions in this retrospective study because comprehensive clinical data were not available. Our data demonstrate that G6 strains were not a major cause of rotavirus disease during the period investigated, a finding that supports previous reports suggesting that these strains do not have a high potential for spread in the population. Nonetheless, the relative frequency of G6 strains (1·4%) in this study compared with some other common serotypes, G3 (1·5%) and G4 (1·5%), in Hungary suggests that it will be important to monitor these infections in the future. To investigate the

epidemiological significance of G6 rotaviruses, it will be necessary to conduct surveillance in additional regions of this country over a longer period.

These findings may also have implications for potential vaccination programmes in Hungary. Current experimental vaccines do not include G6 strains. Thus, it will be important to monitor vaccine efficacy against these strains as well as the impact of a large vaccine programme on their prevalence and epidemiological features to determine the need for a G6 vaccine strain.

Our data suggest that G6 strains have recently emerged or are newly recognized in Hungarian children, raising questions about their origin. Since G6 strains are very common in cattle, one possibility is that human G6 rotaviruses arose by inter-species transmission of bovine rotaviruses to humans or by inter-species transmission accompanied by reassortment. Several studies indicate that previously characterized G6 human rotaviruses contain bovine and human rotavirus genes, supporting the latter hypothesis [19, 22]. This mechanism appears to be very important in the evolution of a variety of human rotaviruses, but direct evidence for it is lacking [37, 38]. Whether, these events occur primarily in those settings where people and animals live in close contact has not been formally demonstrated. We plan to characterize the current G6 strains in more detail to determine if their origin is similar to previously described strains.

As indicated by their RNA electropherotypes and preliminary sequence data, the G6 strains in this study might represent 3 distinct lineages that did not co-circulate but, instead, were detected in consecutive seasons with the exception of season 1996/7, when 2 samples with different electropherotypes one each with profile A and B were detected. We are currently investigating whether these variants have genetic and antigenic differences as indicated by their differing reactivity with MAbs or whether they may represent independent reassortants with other rotaviruses, such as bovine strains.

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