Distribution of Rotavirus VP7 Serotypes and VP4 Genotypes Circulating in Sousse, Tunisia, from 1995 to 1999: Emergence of Natural Human Reassortants

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Rotavirus infection is estimated to result in almost one million deaths of young children in developing parts of the world annually (2). Rotavirus vaccine strategies are based on the need for a polyvalent vaccine candidate encompassing the four epidemiologically important rotavirus VP7 serotypes (G1 to G4), which are commonly detected (6, 13, 26).

The outer capsid of rotaviruses is made up of the VP7 glycoprotein and the VP4 hemagglutinin protein. Both independently induce protective neutralizing antibodies, thus classifying rotaviruses into G (VP7) and P (VP4) serotypes (11). VP7 studies worldwide have identified serotypes G1 to G4 as important, and they are therefore the target for vaccine development (13). G1 strains are most often detected (6, 26). Three human VP4 genotypes (P[8], P[4], and P[6]) are considered significant (6). The VP4 genotypes P[8] and P[4] have been recovered from symptomatically infected children. The P[8] genotype is generally associated with rotavirus strains with VP7 serotypes G1, G3, and G4, while the P[4] genotype is associated with the VP7 serotype G2 (12). The third genotype, P[6], is usually associated with asymptomatic infection in neonates and has been associated with all four VP7 serotypes (12).

In limited North African studies, rotavirus has been reported as a significant etiological agent of severe dehydrating diarrhea in young children. In Egypt, the epidemiology of rotavirus infection was reported to occur in the cooler months of the year and in very young infants and was more significantly associated with watery diarrhea and vomiting as presenting features (3). An early study to characterize Egyptian rotaviruses showed that subgroup II (SG II) strains were more common than SG I strains (14). The most recent study, which analyzed the rotavirus VP7 serotype with monoclonal antibodies, showed that serotypes G1 and G4 predominated and that mixed G1-G4 strains were common (17). Furthermore, G8P[14] strains have also been recently identified in Egyptian children (10). Rotavirus infection has also been reported in North Africa from Algeria (16, 24) and Morocco (23), although no characterization of the rotavirus strains was performed.

In this study, we conducted the characterization of the human rotaviruses found in Sousse, Tunisia, between 1995 and 1999.

MATERIALS AND METHODS

Patient sample. Fecal specimens were collected between February 1995 and May 1999 from 375 infants and young children in Sousse, Tunisia. The specimens were collected from infants and young children between 1 month and 60 months of age presenting with acute diarrheal illness to the Hopital Farhat Hached. Rotavirus detection was performed on 10 to 20% suspensions of the fecal specimens in phosphate-buffered saline, using a latex agglutination assay (Rotavirus Slide; BioMerieux, Marcy l’Etiole, France). The test was performed as specified by the manufacturers.

Polyacrylamide gel electrophoresis. The rotavirus-positive fecal specimens were analyzed by polyacrylamide gel electrophoresis (PAGE) to identify the rotavirus strains in circulation. In brief, the 10 to 20% fecal suspensions were mixed with an equal volume of phenol-chloroform to disrupt the viral particles and release the viral double-stranded RNA (dsRNA) genome (19). After centrifugation at 1,200 × g for 5 min, the aqueous phase containing the dsRNA was precipitated in absolute ethanol overnight at −20°C. Following centrifugation, the dsRNA pellet was resuspended in 0.01 M Tris-EDTA buffer for electrophoresis in 10% polyacrylamide slab gels at 100 V for 16 to 18 h at room temperature. The gels were stained using silver nitrate, as described in detail previously (19).

VP6 monoclonal antibody ELISA. The VP6 subgroup specificity of the rotavirus strains was determined by direct sandwich enzyme-linked immunosorbent assay (ELISA) utilizing monoclonal antibodies that are specific for SG I rotavirus (NIH hybridoma 255/60/125/14) and SG II rotavirus (NIH hybridoma 631/9/104/5b). These monoclonal antibodies have been described in detail elsewhere (8, 9) and have been extensively used in studies in our laboratory as described previously (20). In brief, microtiter plates were coated with a rabbit antiviral serum (20) diluted 1:5,000 in a carbonate-bicarbonate buffer (pH 9.8) and incubated overnight at 4°C. The rotavirus-positive suspensions were added in duplicate to the plates and incubated at 4°C overnight (20). A 1:5,000 dilution of the monoclonal antibodies was added and incubated for 2.5 h at 37°C to allow binding to the appropriate antigens. A conjugated horseradish peroxidase (TMB enzymatic kit; Roche) was used to detect the presence of the subgroup antigen, and the enzymatic reaction was read at 450 nm.

VP7 serotyping assays. Two panels of VP7 serotype-specific monoclonal antibodies were used in this survey. First, VP7 serotype-specific monoclonal antibodies developed by Taniguchi et al. (22) were utilized; these included monoclonal antibodies KU-4 (specific for G1), S2-G10 (for G2), YO-I2E (for G3), and ST-2G7 (for G4), which were kindly donated by Koki Taniguchi to the MRC Diarrhoeal Pathogens Research Unit, MEDUNSA. The second panel of VP7 monoclonal antibodies included monoclonal antibodies specific for serotypes G1 (5E8), G2 (1C10), and G3 (159). A VP7-specific monoclonal antibody (MaH60) was included as a VP7 control. This panel of monoclonal antibodies was supplied by Dennis Lang, National Institutes of Health, Bethesda, Md. The monoclonal antibodies were utilized as described in detail elsewhere (15, 18).

In brief, the microtiter plate strips were coated overnight at 4°C with a 1:5,000 dilution of the monoclonal antibodies in phosphate-buffered saline (pH 7.2), except for 5E8 and 159, which were diluted to 1:10,000. After washing, the plates...
were blocked with 2.5% skim milk (Carnation) in phosphate-buffered saline before the stool specimens were added for incubation overnight at 4°C. After washing the plates, a 1:10,000 dilution of a rabbit antirotavirus hyperimmune serum (kindly donated by Taka Hoshino, National Institute of Allergy and Infectious Diseases) was incubated at 37°C for 1 h to bind to the captured rotavirus particles. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Dako) was added as the detector antibody, and the presence of the enzyme was measured spectrophotometrically at 450 nm using the TMB enzymatic kit (Roche) as the substrate.

The untyped specimens were analyzed by the nested PCR-typing method of Gouvea et al. (7), which has been widely utilized for genotyping the VP7 gene of human rotaviruses (4, 25).

VP4 genotyping. The VP4 genotype of the rotavirus strains was determined in selected numbers by the typing method devised by Gentsch et al. (5). Initially, the viral RNA was extracted by a phenol-chloroform method, followed by ethanol precipitation and then purification by RNAid (Bio 101 and Southern Cross Biotechnology). The purified RNA was reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega) at 43°C for 25 min in the presence of primers to the VP8* region of the gene (con2 or con3). The cDNA was amplified with the same primers in a Perkin-Elmer thermal cycler (4800) at 95°C for 1 min to denature the cDNA, 48°C for 2 min to anneal the primers, and then 72°C for extension of the strands. A nested PCR was then performed using separate primers specific for regions of the VP8* gene which are divergent in the distinct VP4 gene alleles of rotavirus, but conserved within members of the same genotype (5). The commonly identified human rotavirus VP4 genotypes were included (i.e., P[8], P[4], P[6], P[9], and P[10]).

RESULTS

Overall, rotavirus antigen was detected in 65 of 375 stool specimens (17.3%) collected from infants and young children who presented with diarrhea to the Hopital Universitaire in Farhat Hached, Sousse, Tunisia.

Second, rotavirus infection was found to occur predominantly in the cooler season in Tunisia, with most cases occurring between November and February (Fig. 1). Third, all but three of the rotavirus-positive children were less than 2 years of age, with the majority (92%) under 12 months of age (Fig. 2).

PAGE. PAGE was performed on all the rotavirus-positive specimens to examine the genomic diversity of the dsRNA of the strains. In total, 52 of the 65 rotavirus-positive specimens (78%) yielded an RNA electropherotype and showed a limited number of rotavirus strains circulating in the city. There was a major, long RNA electropherotype that circulated every year during the study, with five minor long variations present. In 1999, one of these strains appeared in numbers equal to those of the predominant strain of 1995 to 1998. Only two specimens with a short RNA electropherotype were detected, one each in 1998 and 1999.

Three specimens were seen which carried more than the 11 dsRNA segments, and they were classified as dual infections with more than one strain of rotavirus. Two of these were dual infections between a long and a short RNA electropherotype. VP6 SG specificity. VP6 SG II strains were identified in 50 cases (77%) and predominated overall, as was expected from the overwhelming majority of long RNA electropherotypes seen. In fact, only three SG I strains were identified during the study. Furthermore, two of the SG I strains were identified as G2P[4] strains. In two more cases both SB I and SB II monoclonal antibodies reacted in obvious mixed infections, where strains with both long and short RNA electropherotypes were observed (Table 1). Additional specimens reacted with the group A-specific monoclonal antibody but did not show reactivity with the subgroup-specific monoclonal antibodies.

VP7 serotype specificity. In total, 51 of the strains were serotyped by the monoclonal antibodies used in this study or genotyped by a nested PCR assay for the VP7 serotype. In

FIG. 1. Seasonal distribution of rotavirus infection showing the cumulative percentage of shedding by month in Sousse, Tunisia (monthly cumulative percent rotavirus positive).
1995 to 1997, VP7 serotype G1 viral strains were found to be predominant and were detected in 25 of 34 (73%) of the specimens (Table 1). Serotype G4 strains were detected in four cases, one of which was a dual infection with a G1 strain, which was confirmed by the presence of more than 11 RNA segments in the gel. However, G4 strains became more common during 1999 with the emergence of a new electrophoretic strain. Only two G2 serotypes were seen, one in each of the last 2 years of the study. In 1996 and 1998, G1-G2 mixed infections were detected. No serotype G3, G8, or G9 strains were seen, although 14 strains (42%) could not be typed by the monoclonal antibodies.

**VP4 genotype.** The VP4 genotype assay was performed on 25 selected strains from different RNA profiles. P[8] was the predominant gene allele circulating in Sousse during the study and comprised 52% of the strains identified. The second most commonly seen VP4 genotype was P[6], which was associated with G4 strains in 1995 and 1999. VP4 P[4] was detected in the G2 specimens in 1998 and 1999 (Table 1). VP4 P[4] genotypes were detected more commonly during the last year of the study, but were associated with a G1 VP7 serotype.

**DISCUSSION**

In this study, we report the first antigenic and genotypic characterization of group A rotaviruses in Tunisia. First, group A rotaviruses were detected in the stools of 17% of the infants and young children who presented with acute diarrheal disease to the Hopital Farhat Hached. This figure is lower than the 40% reported in Egypt (3) or 33% in Sicily (1). However, the prevalence of rotavirus infection in this study is similar to that reported in other countries in North Africa, such as Algeria (16, 24), Morocco (23), and, in an earlier study, Egypt (17). In the present study, rotavirus infection was detected by a latex agglutination assay, compared to the ELISA techniques used elsewhere, and this may account for the generally slightly lower levels of rotavirus detection observed. This has been described previously (21).

Second, rotavirus infection was found to occur predominantly in the cool, dry season in Tunisia. This is also similar to the pattern seen in other North African countries where rotavirus infection has been recorded, such as Morocco (23), Algeria (16, 24), and Egypt (3), where epidemiological studies showed the same seasonal trend. Third, this study confirms that rotavirus infection in this region occurs in children less than 2 years of age, and predominantly in infants less than 12 months of age, as described for Egypt (17). Interestingly, in other Mediterranean areas and countries, such as Sicily (1) and France (5), rotavirus infection has shown similar trends, peaking in the cooler months of the year and predominantly infecting younger children.

PAGE of the rotavirus RNA showed the presence of limited strains circulating in the city. A single, long RNA electrophoretic pattern predominated and was seen in 63% of all cases. This strain also persisted throughout the 5 years of the study, although in 1999 another long RNA electropherotype was detected commonly. In addition, only two short RNA patterns were seen in stool specimens, indicating an unusual absence of these strains over a relatively long period of time. Two mixed infections with long and short electropherotypes were observed. This contrasts with the situation described in Casablanca, Morocco (23), where in just 1 year, nine different...
RNA electropherotypes were observed, with a clear shift noted during the year.

The apparent lack of genomic diversity, which would be expected in an urban setting, may be due to the limited numbers included in this study. Nevertheless, minor RNA genome patterns were seen each year and one appeared to be competing successfully by the last year of the study.

SG II rotaviruses were identified commonly, as was to be expected. Other studies have reported a predominance of VP6 SG II strains in this region, in Egypt (14), in Sicily (1), and elsewhere (6, 20, 27).

Reports of the distribution and epidemiology of the human rotavirus VP7 serotypes and VP4 genotypes in North Africa are limited in number. In this study, the vast majority of strains were typed as G1P[8] strains, with G4P[6] and G1P[6] strains reflecting both the earlier study performed in Egypt (17) and the trend seen globally (6). In the only similar study undertaken in North Africa, where the VP7 serotype of rotaviruses in Egypt was examined, G1 and G4 strains also predominated and G1-G4 mixed strains were detected quite commonly (17). In recent studies in France (5) and Sicily (1), G4 strains were most common.

In this study, a few apparently “reassortant” viruses were identified which carried a G1P[4] background. Most human rotaviruses show a strong association of the G1 serotype with a P[8] VP4 genotype, and the P[4] genotype is normally determined in viruses with a G2 VP7 serotype (6, 13, 26). These G1P[4] reassortant rotaviruses have been described to occur at a low level in other settings, such as in Sicily (1), the United States (5), and Japan (27). It was surprising that although G2P[4] strains occurred in such low numbers in Sousse during this study, there were two dual infections with a G2P[4] and G1P[8] strain. It is unclear why this should be so. Nevertheless, the potential for reassortment between these two viruses to generate the G1P[4] strain is clear from the number of these reassortants found in 1998–1999. Other studies have also demonstrated the potential for human rotavirus reassortants, which in some areas appear to occur at high levels (25).

Further studies to determine the VP7 serotype of circulating strains of rotaviruses are needed in developing countries for a number of reasons. First, the recent development of an effective group A rotavirus vaccine, although withdrawn from use by the company currently, has stimulated further research for a rotavirus vaccine. However, the successful implementation of a vaccine requires an understanding of the VP7 serotype epidemiology of rotaviruses in Africa. This study is the first to report the VP7 serotype and VP4 genotype of circulating rotavirus strains in Tunisia; however, further studies are planned to investigate the epidemiology of human rotavirus VP7 serotypes in North Africa.

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