Rotavirus Strain Diversity in the Centre Coast of Tunisia from 2000 through 2003

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An epidemiological survey investigating rotavirus infection in children was undertaken in the coastal region of Tunisia from January 2000 through September 2003. A total of 309 fecal specimens were screened by enzyme-linked immunosorbent assay and latex agglutination assay for the presence of group A rotavirus antigen. The detection rate was 26.2%. Rotavirus outbreaks showed a temperature-dependant pattern ($P = .026$) but no significant association with rainfall. Rotavirus strains isolated were analyzed by RNA polyacrylamide gel electrophoresis and were characterized antigenically by monoclonal antibodies to the VP6 subgroup. Eight RNA electropherotypes were identified, with 3 long and 5 short different RNA profiles. Among VP6 typeable strains, all isolates with a long electrophoretic pattern carried the subgroup II specificity, whereas those with a short profile belonged to subgroup I. In total, 48 rotavirus-positive samples were analyzed for G and P typing by reverse-transcription polymerase chain reaction. A total of 8 different G and P combinations were found: G1P[8] (35.7%), G1P[6] (21.4%), G2P[4] (4.8%), G3P[4] (4.8%), G4P[6] (4.8%), G8P[8] (4.8%), G3P[8] (2.3%), and G4P[8] (2.3%). Mixed infections were detected in 19.1% of stool samples. The emergence in Tunisia of unconventional types, such as G8VP7 specificity, highlights the need for a continual survey of the uncommon strains in North Africa.

Group A rotavirus strains are the most important cause of gastroenteritis in preschool children worldwide. They are responsible for 20%–70% of hospital admissions for acute gastroenteritis in children <5 years of age, and they account for an estimated 611,000 deaths each year [1]. These virus strains belong to the rotavirus genus of the Reoviridae family and are classified serologically in 7 distinct groups (A–G). Group A rotavirus strains are the most commonly found rotavirus strains in acute infantile diarrhea.

The rotaviral genome is formed by 11 double-stranded RNA (dsRNA) segments encapsulated by a triple-layered capsid [2]. Analysis of the electrophoretic mobility of the 11 segments by polyacrylamide gel electrophoresis (PAGE) yields a pattern that is both constant and characteristic for a particular rotavirus isolate. The group A rotavirus strains can be further characterized antigenically by the presence of a subgroup antigen that forms part of the inner capsid of the virion and that is encoded by gene segment 6. Four subgroups (I, II, I+II, and non-I/II) have been observed among group A rotavirus strains. In general, human strains with long electropherotypes (faster-moving gene segment 11) have been found to be associated with subgroup II specificity, whereas strains with short electropherotypes (slower-moving gene segment 11) usually exhibit subgroup I specificity [2].

The incidence and distribution of human group A

rotavirus genotypes varies among geographical areas during a rotavirus season and from one season to the next. Because VP7 and VP4 genes can and do segregate independently, a dual typing system is necessary to characterize the strains of rotavirus that are cocirculating during different seasons in different locations [2]. This system is based on the major outer capsid glycoprotein (VP7), which encodes the major neutralization antigen, determining the G type, and the outer capsid spike protein (VP4), which is protease sensitive and determines the P type [2]. Both elicit neutralizing antibodies in the host and are considered to be important for vaccine development [2].

In the present study, we report the molecular characterization of rotavirus strains by examining the VP6 subgroup specificities, RNA electropherotypes, and the VP7 and VP4 genotypes of rotavirus strains that circulated in the Centre Coast of Tunisia from 2000 through 2003.

MATERIALS AND METHODS

Study design. A total of 309 fecal specimens were collected from children <5 years of age from January 2000 through September 2003. All children came from the Tunisian Centre Coastal region around the cities of Sousse, Monastir, and Mahdia. The children were either outpatients consulting for gastroenteritis or patients who were hospitalized at one of the regional university hospitals: Farhat Hached (Sousse), Fattouma Bourguiba (Monastir), and Tahar Sfar (Mahdia).

All samples were immediately screened for the presence of group A rotavirus antigens. Rotavirus-positive samples were stored at −80°C until molecular analysis. Meteorological data of the Centre Coast of Tunisia were provided from the Regional Office of Monastir of Agricultural Development (Agriculture Ministry of Tunisia).

Detection of group A rotavirus antigens. All samples were processed for rapid diagnosis by latex agglutination assay (Rotavirus Slidex; Biomerieux) and direct sandwich enzyme-linked immunosorbent assay (ELISA; IDEIA Rotavirus; Dako). These tests were conducted and interpreted according to the manufacturer's instructions.

PAGE. Rotavirus-positive fecal specimens were analyzed by PAGE to identify the rotavirus strains in circulation. In brief, fecal suspensions (10%–20%-suspensions of the fecal specimens in phosphate-buffered saline) were mixed with an equal volume of phenol-chloroform to disrupt the viral particles and release the viral dsRNA genome. After centrifugation at 1200× *g* for 5 min, the aqueous phase containing the dsRNA was precipitated in absolute ethanol overnight at −20°C. After centrifugation, the dsRNA pellet was resuspended in 0.01 mol/L Tris-EDTA buffer for electrophoresis in 10% polyacrylamide slab gels at 100 V for 16–18 h at room temperature. The gels were stained using silver nitrate, as described in detail elsewhere [3].

Subgroup assay. The VP6 subgroup specificity of the rotavirus strains was determined by direct sandwich ELISA using monoclonal antibodies raised against the subgroup I and II antigens. These monoclonal antibodies have been described in detail elsewhere and have been extensively used in studies in our laboratory as described elsewhere [3, 4]. In brief, the wells of microtitre plates were coated with a rabbit antirotavirus serum diluted 1:5000 in a carbonate-bicarbonate buffer (pH, 9.8) and were incubated overnight at 4°C. The rotavirus-positive suspensions were pretreated with EDTA to strip off the outer capsid of the rotavirus and reveal the subgroup-specific protein. These pretreated stool samples were then added in duplicate to the plates and incubated overnight at 4°C. A 1:5000 dilution of the monoclonal antibodies to subgroup I, subgroup II, and the group A antigens 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/VP4 genotyping. RNA was extracted and purified from 100 μL of 10% fecal suspensions in phosphate-buffered saline with use of the TRIzol method (Gibco). The extracted RNA was used for seminested multiplex reverse-transcription polymerase chain reaction (RT-PCR) after specific priming with VP7 and VP4 consensus primer pairs, as described in detail elsewhere [5, 6]. In brief, full-length VP7 gene products were obtained with a mixture of the primer pair *sBeg9/End9* and then G typed using the type-specific primers described by Gouvea et al [5]. In the case of P typing, the 876-bp fragment, which includes the VP8* hypervariable region, was amplified with the consensus primers *Con2* and *Con3* and then P typed with the specific primers described by Gentsch et al [6]. All amplified products were examined by gel electrophoresis in 1.2% agarose gels containing 4 μg/mL ethidium bromide under standard conditions.

Statistical analysis. The monthly rate of rotavirus detection was compared with the mean monthly temperature and rainfall in the studied region with use of the Statcalc Program Epi Info, version 6.0 (Centers for Disease Control and Prevention). Correlations were assessed using Pearson's correlation coefficient, and levels of $P < .05$ were used.

RESULTS

Rotavirus detection rate. A total of 309 single samples from children <5 years of age were screened for rotavirus in this study. Of these, 81 (26.2%) were positive by ELISA and/or by latex agglutination assay.

Seasonality. The duration of the rotavirus epidemic season changed from year to year, and the peak incidence in the outbreaks varied from October to March (Figure 1). Although prevalence was higher in the winter, as is common in temperate
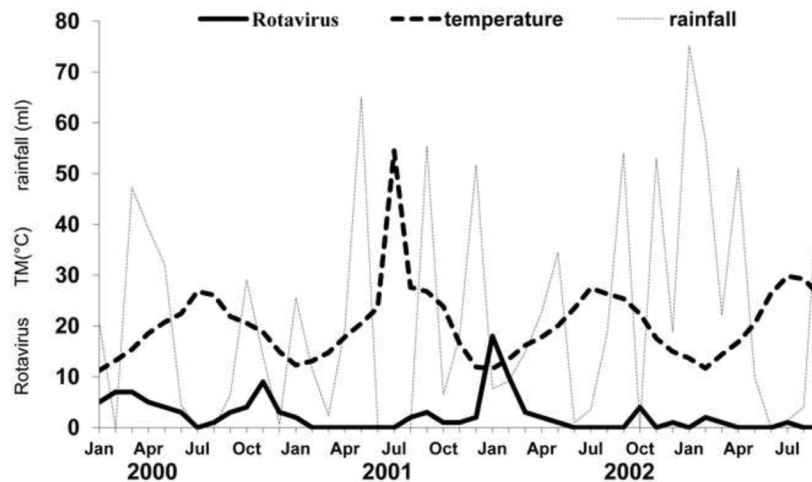
Figure 1. Mean monthly detection of rotavirus, temperature, and rainfall in the Centre Coast of Tunisia, 2000–2003.

climates, the virus also circulated at low levels throughout the summer during 2000, 2001, and 2003. The monthly distributions of rotavirus detection, the mean temperature, and rainfall for the Central Coastal region of Tunisia during the study period are shown in Figure 1. Rotavirus infection appeared to inversely correlate with temperature (Pearson correlation coefficient, -3.58; $P = .016$), whereas there was no statistically significant correlation with rainfall.

Molecular characterization of rotavirus strains by PAGE. A typical group A rotavirus RNA electropherotype was observed in 63 of 81 stool specimens positive for rotavirus by enzyme immunosorbent assay. For the remaining strains there was either not enough stool sample for the analysis or insufficient RNA in the sample for detection by PAGE. Rotavirus strains generally possess either the short or long RNA electropherotype. In this study, 8 different RNA electropherotypes were identified, with a variety of RNA long profiles in 3 cases (labelled L1–L3) and varying short profiles in 5 cases (S1–S5). Short electropherotypes were identified during the first year of the study exclusively and coexisted during this period with 1 long electropherotype (L1). Electropherotypes L2 and L3 appeared in 2001, and L2 became the predominant strain in the region. Only 1 strain, as defined by the L1 electropherotype, persisted during the whole study period.

VP6 subgroup analysis. In the present study, 8 of the 81 specimens positive for rotavirus by enzyme immunosorbent assay could not be characterized by the monoclonal antibodies for the VP6 antigen. Nevertheless, all typed rotavirus strains with short electropherotypes carried the VP6 subgroup I specificity, whereas strains with long electropherotypes exhibited VP6 subgroup II specificity.

Genotyping results. A total of 44 of the 63 strains were genotyped using a nested RT-PCR assay for the VP7 genotype. G1 viral strains were found to be predominant and were de-

tected in 32 specimens (72.7%). G3 and G4 strains were detected in 3 cases each (6.9% each), and G2 and G8 were detected in 2 cases each (4.5% each). Two mixed strains were found: G(1+3) and G(1+4).

A total of 42 strains were VP4 typed using a nested RT-PCR assay. P[8] was the predominant gene allele circulating on the coast of Tunisia during the study and comprised 40.5% of the strains identified. The second most frequently seen VP4 genotype was P[6], detected in 13 cases (30.9%). VP4 P[4] was found in 6 specimens (14.3%). A high rate (14.3%) of mixed VP4 types was noticed: 4 strains P[4+6], 1 P[4+8] and 1 P[6+8].

A total of 39 strains were both G and P typed. Eight different G and P combinations cocirculated in Tunisia during 2000–2003, reflecting the high level of genomic diversity. Moreover, 19.1% of mixed infections, identified by the presence of different G and/or P types in the same sample, were detected. Results of G and P combinations are summarized in Table 1.

DISCUSSION

Rotavirus was detected in 81 stool samples (26.2%) from children <5 years of age in the present study. This detection rate is higher than the rates (17%–22.5%) reported in the previous Tunisian studies [3, 7, 8]. However, the prevalence of rotavirus infection in the present study was similar to those found in many other studies, including similar figures in South Africa and Italy [9, 10], although it is still lower than those seen in developing countries in sub-Saharan Africa [11].

There were variations in the weeks during which the annual epidemic rotavirus season was observed in this study, although it occurred in general at the end of the calendar year from October through January [3]. In the 2000/2001 rotavirus season, however, it ran from December to March and was associated with a higher than usual prevalence (Figure 1). This was

Table 1. VP7 and VP4 Genotypes of Group A Rotavirus Strains Isolated in the Centre Coast of Tunisia from January 2000 through September 2003

VP7/VP4 genotype	No of cases (%)[a]					
	P4	P6	P8	P mixed	P untyped	Total
G1	0	9 (21.4)	15 (35.7)	6 (14.3)	2	32
G2	2 (4.8)	0	0	0	0	2
G3	2 (4.8)	0	1 (2.3)	0	0	3
G4	0	2 (4.8)	1 (2.3)	0	0	3
G8	0	0	2 (4.8)	0	0	2
G mixed	0	2 (4.8)	0	0	0	2
G untyped	0	0	0	0	19	19
Total	4	13	19	6	21	63

[a] Percentages of corresponding genotypes among the 39 successfully both G- and P-typed strains.

associated with the emergence of the L2 strain, which was typed as G1. It was notable that in the following season that began in October, 2001, the prevalence was unusually low, and the same G1 strain, indicated by its L2 electropherotype, predominated.

Furthermore, the seasonal peaks of rotavirus infection analyzed in this study, as in others [9–12], showed a temperature-dependent pattern; rotavirus infection had a seasonal pattern, with epidemic peaks occurring during the cooler months of each year. Previous studies showed that, in temperate climates, rotavirus gastroenteritis tends to be very common in winter and uncommon to virtually absent in summer [13]. In tropical areas, rotavirus infection is common year round, but the prevalence seems to increase during periods of low rainfall or low humidity or decrease during periods of high rainfall and/or high humidity. The cause for this striking seasonal pattern is not well known, but several hypotheses have been suggested. The cold-weather effect could be explained as one that both encourages the family to stay indoors in a tightly closed residence (where contaminated air might readily be breathed and contaminated surfaces might readily be touched) and lowers the indoor relative humidity as outside air is brought in and heated. An important effect of relative humidity might be on aerosol formation or particle size [14, 15].

Electrophoresis of the rotavirus genome yielded an RNA profile in 63 of the 81 rotavirus-positive samples, and 8 different electropherotypes were identified. One of these strains, L2, was predominant and accounted for 33 cases, and minor variants also circulated. This pattern of simultaneous circulation of several different rotavirus electrophoretic strains with a single type predominating is typical of rotavirus epidemiology [9]. Comparison of the profiles found in the present study with those reported in the previous Tunisian report investigating electropherotypes [4] showed that the electropherotype L1 corresponded to the predominant profile circulating during 1995–

1999 in the same geographical area, indicating a shift from L1 to L2 during 1995–2001.

As expected for human rotavirus strains, all strains with long RNA electropherotypes exhibited VP6 subgroup II specificity, and those with short profiles had a subgroup I specificity [9]. Of the 8 specimens that could not be typed as either subgroup I or II, 6 were positive for the group A antigen. Most of these isolates did not react with the subgroup monoclonal antibodies and may represent members of the third subgroup of human rotavirus non-I/non-II. The remaining 2 specimens that could not be typed were negative for the group A antigen control, indicating a lack of sufficient antigen present in the specimen.

In the present study, as in previous studies undertaken in this region of Tunisia [3, 7], genotype G1 predominated, and mixed strains were detected quite frequently during 1995–2004 [7]. Another Tunisian study conducted during 2003 and 2005 found that genotype G3 predominated during that period [8]. Only 3 cases (6.9%) of strains with G3 specificity were found in the present study investigating samples collected during 2000–2003, suggesting that G3 strains quickly increased during the latter period. These temporal fluctuations of the predominant strains of rotavirus have been reported elsewhere [7, 11, 20, 21, 25].

In this study, we also detected 2 cases (4.5%) of the uncommon G8 genotype, which has been reported in developing countries in Africa and has rarely been seen in industrialized countries [11, 12, 16]. The greater genetic diversity of rotavirus described in developing countries might be attributable to the close relationship between humans and livestock in daily life in certain communities [2]. Indeed, previous studies have shown interspecies transmission of rotavirus strains between various host species, such as humans, cattle, pigs, dogs, cats, and horses. Recently, sequencing data demonstrated that rotavirus is able to cross species barrier [17]. The circulation of unusual animal rotavirus strains in infants in developing coun-

tries (such as G8, which is classically isolated from cattle) reflects the exposure to an environment contaminated with human and animal feces. Supporting this speculation, in 2004, G8 genotype was the most prevalent genotype among Tunisian cattle [18]. Presumably, when rotavirus strains cross the host-species barrier, they are not naturally able to efficiently infect or spread in a new host [2, 17]. Instead, rotavirus strains tend to reassort with other rotavirus strains inherent in that host species, and the resultant reassortant strains may be more biologically efficient for infection and spread among the population of the new host [17].

P[8] VP4 genotypes predominated in Tunisia, as described previously, and this VP4 genotype was associated with VP7 G1 specificity [3, 7, 8]. Nevertheless, in the present study, the frequency of P[6] strains was higher than that observed previously. The VP4 P[6] genotype was associated with G1 VP7 specificity and corresponded with the increase in circulation of L2. P[6] strains, although initially associated with asymptomatic neonatal rotavirus strains [11], have been reported to be associated with virulent wild-type rotavirus strains in older infants, especially in Africa [11, 19, 20].

Finally, rotavirus strains with unusual VP7/VP4 combinations, such as G3P[4], were also observed. Classically, the human G3 rotavirus strains show a strong association with the P[8] VP4 type, and the P[4] type is normally determined in strains with a G2 VP7 serotype [2]. These G3P[4] rotavirus strains have been described to occur at a low level in other African settings, such as Malawi [19]. The isolation of strains with unusual combinations of genes strongly supports the idea that conditions in developing countries favor the emergence of new rotavirus strains. Indeed, there is now strong evidence to suggest that cocirculating rotavirus strains continuously interact genetically by reassortment. Sequencing of the VP7 and VP4 genes of rotavirus strains with common and uncommon G and P combinations provided the evidence of reassortment among cocirculating rotavirus strains [21, 22]. Thus, it has been shown that genetic interaction by reassortment among cocirculating rotavirus strains is not a rare event and contributes significantly to their overall diversity [21].

Reassortment opportunities depend on double infections of cells and hosts. The detection of multiple rotavirus strains in a single fecal specimen maximizes the potential of reassortment events at high rates in natural rotavirus infection [2, 11, 19, 23]. Similar to findings in the present study, mixed infections and putative reassortant strains have been found at high frequency in many countries, particularly developing countries. There is evidence that putative reassortants can spread and become epidemiologically significant strains (eg, G1P[4] in Argentina [24] or G2P[8] in Bangladesh [25]). Thus, even if human rotavirus G1P[8], G2P[4], G3P[8], and G4P[8] strains are seemingly better adapted to the human host in genetic terms,

there is opportunity for the successful introduction and cocirculation of other rotavirus strains [21].

The diversity of strains circulating in different regions may be important for vaccine development and clinical evaluation. Recently, 2 live oral vaccines were licensed to protect children against rotavirus diarrhea: RotaTeq (Merck), a bovine-human pentavalent vaccine, including the VP7 antigens for G1–G4 [26, 27], and Rotarix (GlaxoSmithKline), a G1P[8] monovalent human rotavirus vaccine [28, 29]. Clinical trials of these vaccines among children in industrialized and upper-middle–income countries have shown high efficacy against the most common rotavirus strains [26, 29, 30]. The question remains whether these vaccines will protect as well against rotavirus strains that share neither serotype antigen with the vaccine. These vaccines may protect less well against unusual strains circulating in countries planning to implement a rotavirus vaccine strategy. Therefore, it will be important to continuously monitor rotavirus strains before, during, and after vaccine implementation to evaluate the impact of vaccines on strains and help understand the effect of the strain variation on the efficacies of these vaccines [31, 32].

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