

Characterization of Rotavirus Strains in a Danish Population: High Frequency of Mixed Infections and Diversity within the VP4 Gene of P[8] Strains

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We characterized the G and P types from 162 rotavirus-positive stool specimens collected from 162 persons in Denmark (134 children and 28 adults) with acute diarrhea in 1998, 2000, and 2002. Samples were obtained during outpatient consultations (73%) and from hospitalized patients (27%). Although more than 20 different G-P combinations were identified, only 52% represented the globally most common types G1P[8], G2P[4], and G4P[8]. The G9 genotype, which is emerging worldwide, was identified in 12% of all samples. Twenty-one percent of the samples were of mixed genotypic origin, which is the highest frequency reported in any European population. The standard reverse transcription-PCR methods initially failed to identify a considerable fraction of the rotavirus P strains due to mutations at the VP4 primer-binding sites of P[8] strains. The application of a degenerate P[8] primer resulted in typing of most VP4 strains. There was considerable year-to-year variation among the circulating G-P types, and whereas G1P[8] was predominant in 1998 (42% of samples) and 2002 (26%), G2P[4] was the strain that was most frequently detected in 2000 (26% of samples). Our findings might implicate challenges for rotavirus vaccine implementation in a European population and underscore the importance of extensive strain surveillance prior to, during, and after introduction of any vaccine candidate.

Human group A rotaviruses are the most frequently identified etiologic agents in children hospitalized due to acute, severe, dehydrating diarrhea worldwide. Rotavirus infections range in presentation from asymptomatic mild infections to severe and sometimes fatal disease. An estimated 450,000 children who are less than 5 years old die of rotavirus infections each year, most in developing countries (8, 24).

Rotaviruses are triple-layered icosahedral particles, and their genomes consist of 11 segments of double-stranded RNA. Rotaviruses are classified according to the genetic and antigenic diversity of the two outer capsid proteins, VP4 and VP7. These proteins independently induce type-specific neutralizing antibodies and form the basis of the present dual classification of group A rotaviruses into P (protease sensitive) and G (glycoprotein) subtypes, respectively (22).

Rotaviruses express an extensive antigenic and genomic diversity. To date, 14 G serotypes and 20 P types have been defined (19, 22), but a 15th G serotype and a 21st P genotype based on nucleotide sequence characterizations of a bovine rotavirus strain have been proposed (28). Reassortment of genes can occur upon dual infection of a single cell with two different strains *in vivo* as well as *in vitro*. As the segregations of VP4 genes and VP7 genes occur independently, various G and P combinations have been observed in natural infections (26).

The relative incidence and distribution of rotavirus types varies between geographical areas during a rotavirus season and from one season to the next, a close parallel to the molecular epidemiology of influenza virus. Global reviews of the main G and P type combinations encountered in human infections have identified G1P1A[8] (where P1A is the serotype designation and P[8] is the genotypic designation), G2P1B[4], G3P1A[8], and G4P1A[8] as the most frequent (14); however, other G types (normally associated with animals) are predominant in various geographical settings: G5 (found in pigs and horses) in Brazil (16), G8 (found in cattle) in various parts of Africa (1, 5, 11), and G10 (found in cattle) in India (23). Since 1995, the G9 strain (found in pigs) has infected humans in most continents (7), suggesting a possible emergence of a fifth common G type worldwide.

The main strategy for controlling severe rotavirus disease is through vaccination. Appropriate rotavirus strain surveillance in a community is essential before the introduction of a vaccine, during a vaccine campaign, and at follow-up to monitor the prevalence of the different G and P types circulating in an area and to detect uncommon and novel types which might help explain vaccine failure. An important issue to be addressed in future trials is the level of protection provided against severe diarrheal disease in countries with high prevalences of uncommon strains and mixed infection.

Each year, at least 1,000 Danish children who are less than 5 years of age are hospitalized due to rotavirus diarrhea (9). Only a few recent studies have addressed the epidemiology of rotavirus infections in Denmark (9, 10), but the circulating rotavirus strains have never been identified. In the present

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study, we characterize the G and P types of 162 rotavirus-positive stool samples collected in 1998, 2000, and 2002 from a sample of the Danish population.

MATERIALS AND METHODS

Specimens. In all, 162 stool specimens from children, adults, and elderly persons with diarrhea from different Danish geographical regions were identified as rotavirus positive by use of an in-house enzyme-linked immunosorbent assay at the Department of Virology, Statens Serum Institute, Copenhagen, Denmark (17). Samples were collected during the rotavirus seasons of 1998 ($n = 52$), 2000 ($n = 57$), and 2002 ($n = 53$). In the first two years of the study, the majority of samples were collected from children who were <4 years old and elderly persons who were >60 years old and analyzed at the Statens Serum Institute according to then-current guidelines. After the guidelines were changed in 2002, samples from all age groups were collected for testing.

RNA extraction and genotyping. Double-stranded RNA was extracted by use of guanidinium isothiocyanate and a commercial RNA identification kit (Bio 101, Inc., La Jolla, Calif.) as previously described (15). Genotyping was performed via a two-step standard procedure. For the identification of the G and P types, the sBeg9-End9 consensus primers were used in reverse transcription-PCR (RT-PCR) (6). Subsequently, the sBeg9 consensus primer was used in a nested multiplex PCR together with the 9T1-1 (G1), 9T1-2 (G2), 9T-3P (G3), 9T-4 (G4), and 9T-9B (G9) typing primers (6). RT-PCR for P types using the Con2-Con3 consensus primers was performed, and cDNA was diluted 10-fold to 100-fold before continuing with the standard multiplex PCR including the 2T-1 (P4), 3T-1 (P6), 1T-1 (P8), and 5T-1 (P10) typing primers (13). Nontypeable samples were subjected to a single-locus PCR with the MW8 (G8) primer (4) and sBeg9. Positive and negative controls were used in the PCR.

Cloning and sequencing. Cloning of VP4 and VP7 gene RT-PCR products was performed by inserting either the full-length Con3-Con2 (876 bp) or sBeg9-End9 (1,062 bp) products or genotype-specific PCR products into the TOPO 4 cloning vector (Invitrogen, Breda, The Netherlands). Most of the cloned inserts were cycle sequenced in both directions by use of infrared-labeled M13 primers and the Thermo Sequenase DYEnamic Direct cycle sequencing kit (Amersham Biosciences, Piscataway, N.J.). Sequence reactions were separated by electrophoresis on 66-cm² glass plates by using a LI-COR sequencer (LiCor Biosciences, Lincoln, Nebr.) as previously described (11). However, a few inserts were sequenced in both directions by using nonlabeled M13 primers and the Prism Ready BIGDYE Terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.). Sequence reactions were separated in capillary electrophoresis using the ABI PRISM 3100 genetic analyzer (Applied Biosystems). The sequences were compared against the GenBank database using the BLAST program.

Statistical analysis. A comparison of proportions was done using independence tests. The relations between age groups (0, 1, 2 to 4, 5 to 59, and 60 and above), calendar periods (1998, 2000, and 2002), and genotypes (G1P[4], G1P[4]P[8], G1P[8], G2P[4], G4P[8], G9P[8], others, nontypeable [NT], and partially typed [PT]) were assessed by using contingency tables analyses. We used Poisson regression on the number of observations in cells cross classified by age group, genotype, and calendar period using calendar period, age group, genotype, and interactions therefore as predictors. The relation between hospitalization (yes or no), calendar period, and genotypes was assessed likewise (persons without information concerning hospitalization were excluded from this analysis [$n = 5$]). All tests were performed as standard χ^2 likelihood ratio tests. All statistical analyses were performed using the SAS system for Windows version 8.

Nucleotide sequence accession numbers. The Danish P[8] nucleotide sequences are published in GenBank: AY509913 (V98-8405), AY509912 (V98-1791), AY509911 (V98-1154), AY509914 (V98-1482), AY509910 (V00-2138), AY509909 (V98-2380), AY509908 (V98-893), and AY509913 (V97-8405).

RESULTS

Prevalence of rotavirus G and P types in Denmark. We analyzed 162 rotavirus antigen-positive samples. Of these, 146 isolates were characterized according to G and P specificities; 16 specimens could not be completely characterized (Table 1). Thirteen samples were partially typed (i.e., only one of the two genotypic specificities was obtained; in nine samples, no P type could be identified, and in four samples, no G type was iden-

TABLE 1. Distribution of rotavirus genotypes among 162 samples from Danish patients in 1998, 2000, and 2002

Genotype	Strains with given genotype							
	1998		2000		2002		1998-2002	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Single infections								
G1P[4]	0		4	7.0	3	5.7	7	4.3
G1P[6]	0		0		1	1.9	1	0.6
G1P[8]	22	42.3	9	15.8	14	26.4	45	27.8
G1P[10]	0		1	1.8	0		1	0.6
G2P[4]	3	5.8	15	26.3	8	15.1	26	16.1
G2P[8]	0		4	7.0	0		4	2.5
G4P[8]	9	17.3	1	1.8	3	5.7	13	8.0
G4P[10]	1	1.9	0		0		1	0.6
G9P[4]	0		1	1.8	1	1.9	2	1.2
G9P[8]	3	5.8	5	8.8	4	7.6	12	7.4
Total	38	73.1	40	70.2	34	64.2	11.2	69.1
Mixed infections								
G1P[4]P[8]	2	3.9	9	15.8	6	11.3	17	10.5
G1P[6]P[8]	1	1.9	0		1	1.9	2	1.2
G1P[8]P[10]	2	3.9	0		0		2	1.2
G2G9P[4]P[6]	0		0		1	1.9	1	0.6
G2G9P[4]P[8]	0		0		1	1.9	1	0.6
G2P[4]P[6]	0		0		2	3.8	2	1.2
G2P[4]P[8]	0		2	3.5	0		2	1.2
G4P[4]P[8]	2	3.9	0		1	1.9	3	1.9
G8P[4]P[6]	0		0		1	1.9	1	0.6
G9P[4]P[8]	0		2	3.5	1	1.9	3	1.9
Total	7	13.5	13	22.8	14	26.4	34	21.0
Partly typed strains								
G1	0		1	1.8	1	1.9	2	1.2
G2	0		1	1.8	1	1.9	2	1.2
G4	3	5.6	0		0		3	1.9
G9	1	1.9	0		0		1	0.6
P4	1	1.9	0		0		1	0.6
P6	0		0		1	1.9	1	0.6
P8	1	1.9	0		0		1	0.6
G1G2	0		1	1.8	0		1	0.6
P[8]P[10]	1	1.9	0		0		1	0.6
Not typed	0		1	1.8	2	3.8	3	1.9
Total	7	13.5	4	7.0	5	9.4	16	9.9
Total	52		57		53		162	

tified). Three specimens (2%) were nontypeable (i.e., neither a G nor a P genotype could be identified).

A total of 152 specimens had single G types, and 3 specimens contained mixed G infections according to multiplex PCR results. In addition, eight G9 specimens proved to be G1G9 mixed infections when subsequently analyzed by single-locus PCR using only the G1 and G9 primers. Because single-locus PCR was limited to subsets of the specimens, these findings are not shown in Table 1, as they do not reflect a "true" distribution.

Temporal distribution of rotavirus infections and genotypes in Denmark. Sample collection dates were known for all samples. In Denmark, rotavirus infections are seasonal, with an-

nual epidemics occurring during the winter and spring months (January to April or May) (9). Although there are no standard procedures in Denmark prescribing when to collect a sample for testing from a person with diarrhea, the numbers of collected samples likely reflect the overall annual rotavirus epidemic activity. In all three years, the number of samples collected peaked in March. To examine the association between genotype profile and year, genotypes appearing with a frequency of more than 4% (Table 1) were kept, whereas NT and PT samples were grouped as NT/PT strains and the other strains were grouped as "others." There was a pronounced interseasonal variation among circulating strains ($\chi^2 = 32.5$; $P < 0.01$): overall during the three 1-year study periods, the most prevalent genotype G and P combination was G1P[8] (28%), followed by G2P[4] (16%) and G1P[4]P[8] (11%). However, in 1998, G1P[8] was predominant, constituting 42% of all samples, followed by G4P[8] (17%) and G9P[8] (6%), whereas in 2000, G2P[4] was the most frequently identified genotype (26%), followed by G1P[4]P[8] (16%) and G1P[8] (16%). The 2002 season differed from the earlier years, with G1P[8] being the most frequent genotype (26%), followed by G2P[4] (15%) and G1P[4]P[8] (11%). The G9 types were spread uniformly over the three years of observation.

Sex and age distribution according to rotavirus G and P types. Fifty-seven percent (92 of 162) of the samples originated from males, and no differences were seen according to calendar year. As the samples for the period 1998 and 2000 were routinely collected only from children who were less than 4 years of age and adults who were more than 60 years old, analyses concerning age should be taken with caution. Ninety-three specimens (57%) were from children who were less than 2 years old, and 29 (18%) specimens were from children who were 2 to 4 years of age (Table 2). Rotavirus specimens were also collected among adults who were more than 20 years of age (age range, 22 to 86 years; mean age, 51 years; median age, 48 years; $n = 28$). Overall, 31% of the strains identified in children who were less than 5 years of age were G1P[8], whereas only 9% of individuals who were older than 60 years of age were infected with this strain. In the ≥ 60 years old age group, 36% of the samples were G2P[4], followed by G4P[8] (27%) (Table 2). However, adjusting for calendar period, the genotype profiles did not differ between age groups ($P = 0.99$).

Distribution of G and P types originating from patients treated as outpatients compared with those admitted to a hospital. In all, 43 specimens originated from hospitalized patients. The majority of these were from infants and children who were less than 2 years of age (26 of 43 [60%]) (Table 2). We found no significant difference in the prevalence of specific genotype infections among the hospitalized patients' samples compared to the outpatients' samples (Fig. 1).

Cloning and sequencing of G9 strains. The Danish G9 strains showed high homology (96 and 97%, respectively) with G9 strains MR4710/00 (AY262747) and MR4730 (AY262749) that were published in GenBank by a South African group (25).

Genotyping of incompletely typed P rotavirus strains using a degenerated P[8] primer. As no P types were found during the initial multiplex PCR typing of 20 samples, a subset of seven incompletely typed samples was cloned and sequenced. These strains proved to be P[8] strains with a high

TABLE 2. Rotavirus genotype and hospitalization by age group in 1998, 2000, and 2002

Genotype or hospitalization status	No. and % of strains for patients in given age group ^a											
	0		1		2-4		5-59		60-85		Total	
	n	%	n	%	n	%	n	%	n	%	n	%
Genotype												
G1P[4]	3	10.3	1	1.6	1	3.5	2	6.9	0		7	4.3
G1P[4]P[8]	1	3.5	11	17.2	2	6.9	3	10.3	0		17	10.5
G1P[8]	6	20.7	19	29.7	13	44.8	6	20.7	1	9.1	45	27.8
G2P[4]	6	20.7	9	14.1	5	17.2	2	6.9	4	36.3	26	16.0
G4P[8]	1	3.5	4	6.3	3	10.3	2	6.9	3	27.3	13	8.0
G9P[8]	1	3.5	4	6.3	2	6.9	4	13.8	1	9.1	12	7.4
NT/PT	4	13.8	6	9.4	0		6	20.7	0		16	9.9
Other	7	24.1	10	15.6	3	10.3	4	13.8	2	18.2	26	16.0
Hospitalized												
Yes	12	41.4	14	21.9	8	27.6	8	27.6	1	9.1	43	26.5
No	17	58.6	49	76.6	21	72.4	18	62.1	9	81.8	114	70.4
No information	0		1	1.6	0		3	10.3	1	9.1	5	3.1
Total	29	64	29	29	11	162						

^a Ages are given in years.

homology (98%) to Japanese P[8] strains (GenBank AB039940/Kagawa/90-551) (Fig. 2). Phylogenetic analyses showed that the P[8] primer-binding sites all contained one- or two-point silent mutations, explaining the initial failure of the primers to bind to the P[8] sites. The degenerate P[8] typing primer 1T-1D (5'-3' nucleotides 340 to 356) as designed by Iturriza-Gomara et al. (20) corresponded to the mutations (Fig. 3). Accordingly, the 1T-1D primer replaced

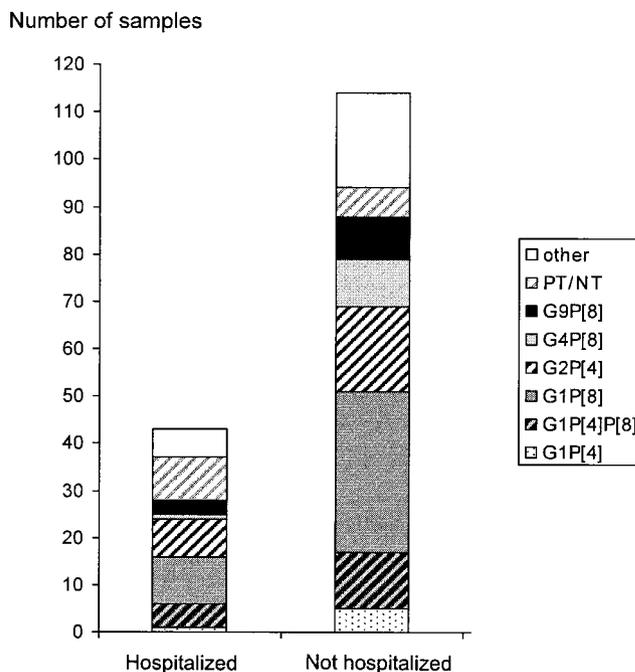


FIG. 1. Rotavirus genotypes in 157 persons in Denmark according to hospitalization status in 1998, 2000, and 2002.

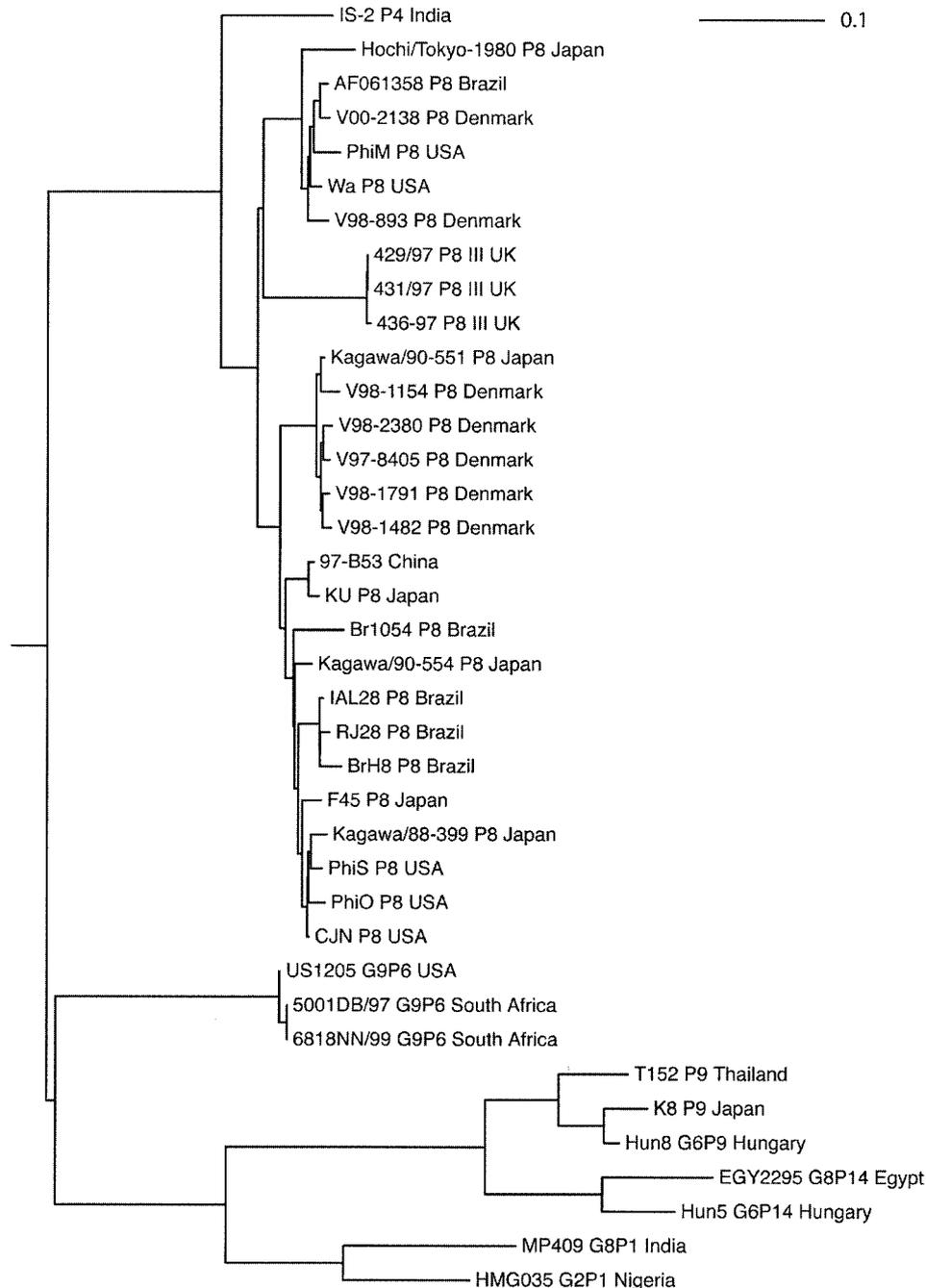


FIG. 2. Consensus tree based on the VP7 genes of a wide selection of P[8] rotaviruses. The tree was elaborated by the neighbor-joining method. The tree is rooted using the VP4 gene of non-P[8] strains.

the original P[8] primer in the standard multiplex PCR, which resulted in typing of most VP4 types.

DISCUSSION

This study represents the first attempt to characterize Danish rotavirus strains. Although more than 20 different G-P combinations were identified, only 52% represented the globally most common types G1P[8], G2P[4], and G4P[8] (14). No

G3 strains were detected. The G9 genotype, which is emerging worldwide, was identified in 12% of all Danish samples, and the uncommon G1P[4] genotype was identified in 4.3% of the Danish samples. As a comparison, this genotype was identified in <1% of rotavirus strains in a study from the United Kingdom (21) over a 3-year period. Other uncommon genotypes identified were G9P[8] (7%), G1P[4] (4%), and G2P[8] (3%). More than 20% of the samples were of mixed genotypic origin, the most common combination being G1P[4]P[8]. The rela-

Primer 1T-1 (rev.comp) GCACGTTATCCAAGTAGA

V98-893_VP4_P8 ...T....A..C.....
 V98-2380_VP4_P8 CGAT.C...
 V97-8405_VP4_P8 A.....CGAT.C.
 V00-2138_VP4_P8 A..C.....
 V98-1154_VP4_P8_C C.AT.C.....
 V98-1791_VP4_P8_C C.AT.C.....
 V98-1482_VP4_P8_C C.AT.CG..

 Primer 1T-1D (rev.comp) CANGTYAAYCCAGTAGA

V98-893_VP4_P8
 V98-2380_VP4_P8 G.....
 V97-8405_VP4_P8 G.....
 V00-2138_VP4_P8
 V98-1154_VP4_P8_C
 V98-1791_VP4_P8_C
 V98-1482_VP4_P8_C G...

FIG. 3. Alignment of fragments of the VP4 gene and the reverse complementary sequences of the original primer 1T-1 and the degenerate primer 1T-1D. The top panel shows Danish strains that were not typed by RT-PCR with the primer 1T-1, and the bottom panel shows Danish strains that aligned with the degenerate primer 1T-1D despite the few sequential mismatches. Residues that match primer 1T-1 and/or 1T-1D are denoted by dots.

tively high frequency of mixed infections detected, 21%, is unusual in a European population. In a 1990 report of rotavirus strains in Austria ($n = 294$) (12), only 2.7% of the samples had mixed genotypes and none were mixed P infections. Similarly, a 1990 study from Great Britain ($n = 2,912$) (21) found that only 2.2% of the samples were of mixed origin. Dual infections are possible when two diverse rotavirus infections occur concomitantly in one individual. Such an occurrence could be a predisposition for reassortment of viruses. We consider it likely that our high yields of mixed infections are due to the modified characterization methods applied, including single-locus PCR as well as cloning and sequencing techniques. The high yield of relatively uncommon strains (e.g., G1P[4], G1P[6], G1P[10], G2P[6], and G9P[4]) corresponds well with these findings.

In developing countries, the high frequency of reassortment reported in several studies (1, 5, 27) is thought to be facilitated by close interactions among humans and among humans and domestic animals. However, in Denmark, close human-animal contacts are primarily related to domestic pets (cats and dogs) rather than farm animals (lambs and pigs). We found no G3 strains (usually detected in cats and dogs) but a relatively high frequency of G9 strains, which are most common in lambs and pigs. After the United States, Denmark is the largest exporter of pork in the world, and the introduction of porcine G9 strains to the human population is a possibility. However, although zoonotic transmission of rotavirus has been suggested (26), it has yet to be proven. We believe that a more likely explanation for the high frequency of dual infections and uncommon types

among Danish rotavirus specimens is underreporting in other studies due to the inability of the standard multiplex PCR assay to detect such infections.

According to our experience, the use of multiplex PCR in the identification of mixed infections should be handled with caution. The possibility of unspecific primer binding has to be taken into consideration. Furthermore, in mixed infections the rotavirus strains might be present at different concentrations, resulting in an uneven degree of PCR amplification, making the interpretation of the gel band pattern difficult (i.e., are there really two bands or just one?). By cloning a few of the multiplex PCR products it was possible to verify the existence of coinfections, and by comparison of these with the full lengths of the VP7 and/or VP4 gene segments, it was furthermore possible to exclude unspecific primer binding as the cause of two bands on this gel (data not shown). We are, however, fully aware of the risk of contamination when handling so many stool samples and conducting similar high numbers of PCR analyses, and we therefore included controls for contamination in our PCR assays.

The standard RT-PCR methods initially failed to identify a considerable fraction of the rotavirus P strains. This problem was solved by cloning and sequencing of a subset of the incompletely typed strains, revealing silent point mutations in the P[8] primer-binding site and explaining the failure of primer binding. The identification of silent point mutations in the primer binding site in P[8] strains has been previously reported by a British group (20), who developed a degenerated P[8] primer. The remaining samples were therefore subjected to a multiplex PCR in which the normal P8 primer was replaced by the degenerated P[8] primer.

Another methodological problem is interprimer suppression, which was reported in our recent study of West African incompletely typeable rotavirus strains (11). The present study also faced this problem: nine rotavirus specimens were initially identified as only G9 strains when subjected to a standard heminested multiplex primer RT-PCR typing using the G serotype-specific primers G1, G2, G3, G4, and G9 in the same mixture. Cloning and sequencing of six of these specimens revealed that four of them were G1 strains, and subsequent PCR typing using the G1 primer alone against the sBeg9 consensus primer identified G1 serotype bands in eight of the nine G9 samples. Furthermore, the repeated findings of interprimer suppression when using the conventional RT-PCR typing procedure underscore the problem of the standard multiplex PCR procedure and the need for regular evaluation and optimization of this procedure.

The finding of a few samples among adults throughout all age groups is also interesting regarding the immunity perspective of this disease, which in general is perceived to be a childhood disease. One likely explanation for the number of rotavirus infections identified among Danish adults is the media attention to fatal *Salmonella* cases, which occurred predominantly among adults in Denmark (18) and created awareness about severe adult diarrhea.

The genotypes identified among the older persons were different in neither type nor frequency compared to the ones identified among the rest of the population. Still, there was a tendency of the G4P[8] strain to be associated with older patients and the G1P[8] rotavirus strains to be associated with

younger patients (Table 2). However, numbers are few, and specimens were routinely collected in 1998 and 2000 only among infants and adults older than 60 years of age.

No specific rotavirus genotype was demonstrated to be significantly associated with rotavirus infections among hospitalized patients compared to patients seen in outpatient settings. In other words, our study does not demonstrate transmission of severe rotavirus infections with specific pathogenic strains within Danish hospitals. In a recent study from Italy ($n = 489$), the P[4] strain was found to be associated with severe infections (3), whereas in several other studies from Switzerland ($n = 553$) (12), Bangladesh ($n = 2,441$) (2), and Mexico ($n = 177$) (29), no such association was demonstrated.

In conclusion, we find it interesting that only about 52% of the identified Danish rotavirus strains are among the four most common rotavirus strains worldwide. Furthermore, we found that 12% of G types identified were G9s and that a surprisingly large fraction (21%) of the strains was of mixed origin. The high fraction of mixed infections might well be explained by the modified technology we have applied, including single-locus PCR, cloning, and sequencing, and highlight the need for a more sensitive characterization tool than that offered by the standard multiplex RT-PCR. The potential impact on vaccine coverage against infections with strains of mixed genotypic origin and/or uncommon or newly emerging strains (i.e., G9) is not yet known and raises important questions to be specifically addressed in future vaccine trials.

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